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ENDOCRINOLOGY

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ANDROGEN EXCRETION IN HUMAN BILE[1]

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PASCHKIS and coworkers (1944) reported the excretion of androgens in the bile of dogs after the intravenous administration of testosterone, androsterone, and methyltestosterone. We have studied the biliary excretion of androgens after testosterone administration in three humans. Longwell and McKee (1942) and Cantarow *et al.* (1942, 1942a, 1943, 1943a, 1943b) have found relatively large amounts of estrogen in the bile of bile-fistula dogs injected subcutaneously with various amounts of estrone and estradiol. The latest report from this group (Pearlman, 1945) indicates a lower recovery of estrogen from the dog's bile than was first reported, but the amount in the bile was still quite significant, 10.4 per cent of the estrogenic activity and 39.3 per cent of the estrogenic material being recovered. Stimmel (1951) has reported recovery of 3 per cent and 7.7 per cent of administered estroil in human bile, and failure to recover any administered estrone or estradiol. We have studied biliary estrogens in one of the three patients.

PATIENTS

The experiments were carried out on three patients. Patient A was a 46 year old woman with jaundice due to a fibrotic constriction in the ampulla

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of Vater, in whom a T-tube was inserted into the common duct. From 350 to 1260 cc. of bile per day were collected in this manner during the course of the experiment. Patient B was a 68 year old man with complete obstruction of the common bile duct due to adenocarcinoma. Complete drainage of bile was obtained with this patient. Patient C was a 61 year old woman with obstruction of the bile duct due to an adenocarcinoma. Studies of serum proteins, cholesterol, bilirubin, icterus index, cephalin flocculation, and prothrombin time indicated that the liver was functioning normally, though there was some jaundice. Complete recovery of bile was obtained from this patient.

EXPERIMENTAL PROCEDURE AND METHODS

Similar experimental procedures were employed for the studies of androgen excretion on all three patients. The designs of the experiments are illustrated in Table 1. One sample of urine and one sample of bile were collected before testosterone propionate was administered to patient A. This patient received orally 600 mg. of testosterone propionate for two consecutive days. Daily urine and bile samples were collected during the period of hormone administration and for the following three days. The experimental

TABLE 1. THE EXCRETION OF ANDROGENS AND 17-KETOSTEROIDS AFTER THE ORAL ADMINISTRATION OF TESTOSTERONE PROPIONATE

Patient (age and sex)	Day of expt.	Testosterone propionate administered, mg.	Bile[a]			Urine	
			Volume, cc.	Androgens, I.U./day	17-KS, mg./day	Androgens, I.U./day	17-KS, mg./day
A (46, F)	1	0	1260	< 2.5	1.3	—	—
	2	600	930	<12.5[b]	0.8[b]	—	97
	3	600	350				215
	4	0	660				11
	5	0	1040	—	1.3[b]	—	7
	6	0	760				4
B (68, M)	1	0	420[b]	—	0.8[b]	—	5[b]
	2	0					
	3	600	580[b]	—	0.8[b]	—	65[b]
	4	600					
	5	0					
	6	0	2000[b]	—	1.6[b]	—	18[b]
	7	0					
C (61, F)	1	0	870	2.5	1.7	—	4
	2	600	640	2.7	0.6	198	25
	3	600	720	3.1	0.9	492	59
	4	0	900	2.8	0.8	156	23
	5	0	800	—	0.8	—	6

[a] The values for biliary excretion reported for patient A are low since only a portion of the bile was collected.

[b] Mean values for daily excretion over the period indicated. Bile or urine samples were combined for extraction.

schedule for patient B was the same as that for patient A except that two days control samples of urine and bile were collected. For patient C collections were made on one control day before testosterone propionate administration, and for two days after testosterone propionate administration.

The urines were acidified with 10 per cent by volume of concentrated hydrocholoric acid. Then 25 per cent by volume of carbon tetrachloride was added, and the mixture refluxed for 6 hours. The carbon tetrachloride was separated from the urine and the urine was refluxed with a similar volume of carbon tetrachloride for another six hours. The combined carbon tetrachloride fractions were evaporated to dryness *in vacuo*, and the residue separated into the neutral and acidic compounds. The neutral fraction was used for the assay of 17-ketosteroids by the method of Holtorff and Koch (1940).

TABLE 2. THE EXCRETION OF ESTRONE, α-ESTRADIOL, AND ESTRIOL AFTER THE ORAL ADMINISTRATION OF α-ESTRADIOL DIPROPIONATE

Patient (age and sex)	Day of expt.	Estradiol dipropionate administered, mg.	Bile and urine extracted with	Bile			Urine		
				Estrone	Estradiol, μg./day	Estriol	Estrone	Estradiol, μg./day	Estriol
C (61, F)	1	0	CCl_4	1.62	0.29	12.0	1.86	0.36	10.8
	5	0	CCl_4	0.82	0.15	12.0	0.58	0.14	16.0
			benzene	1.63	0.28	33.6	12.0	1.29	48.0
	6	100	benzene	18.0[a]	2.13[a]	60.0[a]	410.0[a]	50.0[a]	920.0[a]
	7	100							
	8	0	benzene	10.8	1.78	83.3	440.0	74.0	1760.0
	9	0		2.2	0.71	240.0	105.0	18.5	600.0

[a] Mean values for daily excretion during days 6 and 7. Bile and urine samples were combined for extraction.

The bile was acidified with 15 cc. of concentrated hydrochloric acid per 100 cc. and refluxed with 50 per cent by volume of carbon tetrachloride for three hours. After separating the carbon tetrachloride the acidified bile was re-extracted with another one-half volume of carbon tetrachloride for three hours longer. The carbon tetrachloride fractions were combined and evaporated to dryness *in vacuo*. The neutral fraction was prepared by partitioning the residue between ether and 2.5 N aqueous sodium hydroxide. The neutral fraction was used for the assay of androgens by the method of Frank *et al.* (1942) and for the assay of 17-ketosteroids by the method of Holtorff and Koch (1940).

In order to test the efficiency of our extraction procedure, 20 mg. of dehydroisoandrosterone were added to each of three 250 cc. samples of human bile. There were less than 1.25 mg. of 17-ketosteroids in each of five 250 cc. portions of the same sample of bile. It was possible to recover 87, 91 and 100 per cent of the added dehydroisoandrosterone on the basis of the 17-ketosteroid determinations.

Biliary estrogen excretion was also studied in patient C, according to the design shown in Table 2. On the third and fourth days after the administration of the testosterone propionate, 100 mg. per day of estradiol dipropionate were administered orally, and the urine and bile collected for the days of administfation and the two succeeding days.

The control urine and bile specimens from the first day were extracted with carbon tetrachloride in the same manner as the specimens studied for androgen content. The control urine and bile specimens from the second day were divided into two parts, one part being extracted with carbon tetrachloride and the other having benzene substituted for the carbon tetrachloride. All the remaining urine and bile specimens were extracted with benzene in a manner analogous to that used with carbon tetrachloride.

The estrogen fraction was obtained from the carbon tetrachloride extract by par-

titioning the dried residue between ether and 2.5 N sodium hydroxide, after first washing the ether with saturated sodium bicarbonate to remove the carboxylic acids. The sodium hydroxide extract was acidified with 16 per cent sulfuric acid and re-extracted with ether. Evaporation of the ether gave the combined estrogen fraction.

The estrogen fraction from the benzene extracts was obtained in a different manner. The residues were partitioned between ether and saturated sodium bicarbonate. The ether-soluble substances were taken up in 1 part of ether and 18 parts of carbon tetrachloride. This solution was extracted with 1 N potassium hydroxide (Friedgood, Garst and Haagen-Smit, 1948). The alkaline extract was acidified and re-extracted with ether, yielding the combined estrogen fraction.

Estriol was separated from the combined estrogen fraction by partitioning it between 0.075 M disodium phosphate and an organic phase consisting of one part ethanol to 100 parts benzene (Friedgood, Garst, and Haagen-Smit, 1948). It was recovered from the aqueous phase by acidification with sulfuric acid and re-extraction with ether. Estrone and estradiol were separated in the ethanol-benzene soluble residue by the use of Girard's reagent T (Girard and Sandulesco, 1936).

Estriol, estrone and estradiol were all assayed separately by a bioassay method based on the uterine weight response of the immature mouse (Rubin, Dorfman, Black, and Dorfman, 1951). The activity was determined from a log dose-response curve obtained with crystalline estrogen.

EXPERIMENTAL

The excretion of androgens and 17-ketosteroids after the administration of testosterone propionate is described in Table 1. The values for the bile excretion of patient A are low since only a portion of the bile was collected. However, even after the administration of 1200 mg. of testosterone propionate no increase in the biliary excretion of 17-ketosteroids could be demonstrated. Before the hormone was administered less than 1.3 mg. of 17-ketosteroids were found. In the three day period, which included the two day treatment period, the 17-ketosteroid value was only 0.8 mg. per day. That the hormone was being absorbed was demonstrated by the fact that the urinary 17-ketosteroid titer rose as high as 215 mg. per day during the second treatment day. In all, 31 per cent of the administered testosterone propionate could be accounted for on the basis of the 17-ketosteroid found in the urine.

No androgens could be demonstrated in the bile extracts from patient A. Before hormone treatment a value of less than 2.5 I.U. per day was found in the bile, and during the intensive hormone treatment the androgen assay indicated less than 12.5 I.U. per day, or less than 1.25 mg. of androsterone equivalent. Even if the bile obtained from patient A represented only one-third of the total bile produced, which is a low estimate, the maximum biliary excretion of androgen would be only 4 mg. of androsterone equivalent after the administration of 1200 mg. of testosterone propionate.

In patient B the total bile output was obtained but no real increment in biliary 17-ketosteroid excretion could be demonstrated. The pretreatment

value of 0.8 mg. of 17-ketosteroids remained unchanged after the administration of 1200 mg. of testosterone propionate. Twenty-one per cent of the administered testosterone propionate was accounted for in the urine as 17-ketosteroids.

In patient C, the total bile output was again obtained and again no increment in biliary 17-ketosteroids or androgen excretion could be demonstrated. Control androgen excretion was 2.5 I.U. per day and after administration of 1200 mg. of testosterone propionate it rose only to 3.1 I.U. per day. This represented a 20 per cent increase over the control level. On the same day, the urinary androgen excretion was equivalent to 492 I.U. of androsterone, an increase of 150 per cent over the control level. Bile 17-ketosteroids decreased from a control level of 1.6 mg. per day to 0.6–0.9 mg. per day. Twelve per cent of the administered material was recovered in the urine as 17-ketosteroids.

The excretion of the three estrogens after the administration of estradiol dipropionate is described in Table 2. Benzene is more efficient for extraction of the estrogens than is carbon tetrachloride. After the administration of 200 mg. of estradiol dipropionate, estrone in bile increased from the control value of less than 1.6 μg. to 18 μg. per day for the two days of administration, and was still 11 μg. for the first day after administration, and 2.2 μg. on the second day after administration. Estradiol also increased about 10 times during administration, from the control level of 0.28 to 2.1 μg., and then fell to 0.71 μg. by the second day after administration. The increase in bile estriol was about two-fold during administration (60 μg. per day as compared with the control level of 34 μg.), and estriol excretion continued to rise to 83 μg. on the first day after administration and 240 μg. on the second day after administration, a value of approximately seven times the control level.

Total recovery of estrogenic material in bile was less than one per cent. Urinary estrogen levels increased more strikingly than bile estrogen levels. Estrone increased 34 times during the administration of estradiol and was still elevated by a factor of 10 on the second day after administration. Estradiol increased 38 times during administration, was 57 times the control level on the first day after administration, and 10 times the control level on the second day after administration. Estriol excretion in urine was increased only 19 times during estradiol administration, but was 38 times the control level on the first day and 12 times the control level on the second day after administration.

If we use only the control figures from the benzene-extracted bile and urine it appears that there are approximately 6 times as much estrone in urine as in bile, 4.5 times as much estradiol, and approximately an equal amount of estriol. After the administration of estradiol dipropionate the

ratio of urinary to biliary estrone may be increased as high as 50, estradiol to as high as 41, and estriol to as high as 17.

SUMMARY

The oral administration of 1200 mg. of testosterone propionate during a two-day period to three patients (a 46 year old woman with jaundice due to a fibrotic constriction in the ampulla of Vater, and a 68 year old man and a 61 year old woman who suffered from complete obstruction of the common bile duct due to adenocarcinoma) did not result in an increased biliary excretion of either 17-ketosteriods or androgens. That the administered androgens were absorbed was evidenced by the fact that 31, 12 and 21 per cent of the testosterone propionate administered appeared as 17-ketosteroids in the urines of the two women and the man respectively.

Data obtained after administration of estradiol dipropionate (200 mg.) to one of the patients are in contrast to the findings after testosterone administration. Biliary and urinary excretion of all three naturally occurring estrogens (estradiol, estrone, and estriol) were studied. Urinary excretion of each of the three estrogens increased more than 30 times after estradiol administration. Though daily excretion of each estrogen in bile was smaller at all times than the urinary excretion, bile excretion of each estrogen increased from 8 to 10 times after administration of estradiol dipropionate.

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SIGNIFICANCE OF CHANGES IN RENAL AND HEPATIC L-GLUTAMIC ACID DEHYDROGENASE ACTIVITY IN HYPOPHYSECTOMIZED OR NORMAL RATS TREATED WITH GROWTH HORMONE OR THYROXIN*

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LARGELY as a result of studies by Braunstein and co-workers (1939, 1945), attention has been directed to a pathway of amino acid catabolism in which transaminases and L-glutamic acid dehydrogenase are involved. It seemed of obvious interest to determine whether or not the activity of the latter enzyme in tissues is diminished during growth induced with somatotrophin preparations, for it is conceivable that the observed anabolic effects might be due in part to interference with the oxidative deamination of glutamic acid formed from other amino acids by transamination.

EXPERIMENTAL

For our studies of L-glutamic acid dehydrogenase, normal and hypophysectomized Sprague-Dawley rats of the same age were obtained simultaneously from the same source.[1] Since these animals were also used for studies on tissue cathepsin, details concerning the design of experiments, growth hormone preparation, diet, handling of tissues, and some of the methods of analysis, have already been given (Mathies, Palm, and Gaebler, 1951).

L-glutamic acid dehydrogenase activity was estimated on the day of sacrifice in the case of 4 rats of each group. The tissues of the remaining rat were stored at $-18°$ and tested within the next 4 days. Whole homogenates (Potter and Elvehjem, 1936) of liver and kidney were prepared in double-distilled water at 0° and assayed immediately. The remaining homogenate was frozen on dry ice and stored at $-18°$ for subsequent determinations of total nitrogen, which were carried out in triplicate on pooled whole homogenates from each experimental group.

In all experiments, estimation of L-glutamic acid dehydrogenase was carried out with the Warburg apparatus, by measuring oxygen uptakes at 38°, with air as the gas phase, 0.2 ml. of 10% potassium hydroxide in the center well, and a shaking rate of 120 per minute. The coenzyme I[2] employed was stated to be 65% pure or better. The

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[2] Schwarz Laboratories, Inc., New York, N. Y.

exact procedure varied in different series of experiments, but was of course uniform in each one. At first, the assay employed was one developed from the procedure used by Dewan (1938). Later, the assay of Copenhaver, McShan and Meyer (1950) became available and was used in all subsequent work.

The essential difference between the methods of assay is that different buffers and carriers are involved. In our modified Dewan procedure, the Warburg vessels contained 0.2 ml. of neutralized M/2 glutamate in the side arm and, in the main chamber, 0.5 ml. of 9.1% (1:10) homogenate, 0.5 ml. of 1% neutralized DPN, 0.2 ml. of 0.5% neutralized synthetic carrier, 1 ml. of 0.1 M pyrophosphate of pH 7.6 (25°) prepared fresh daily from 3.5 ml. of N HCl and 25 ml. of 0.2 M sodium pyrophosphate diluted to 50 ml. For liver, 0.3 ml. of 0.1 M neutralized nicotinamide was also added to the main chamber. In the blanks, water replaced glutamate. These digests were equilibrated open for 20 minutes, closed for 10 minutes, and mixed. Readings were taken at 0, 10, and 20 minutes. pH values of digests were between 7.6 and 7.7 at 25°, and the temperature shift of the buffer was such that at 37° the pH was 7.3–7.5. Duplicate assays gave consistently good checks. Activity is expressed as 3 times the 20-minute uptake after correction for the blank. The carriers employed are indicated in the tables. The method of Copenhaver, McShan and Meyer (1950) was used without alteration. Each digest contained 0.2 ml. stock cytochrome *c* (Umbreit, Burris, and Stauffer, 1945) which was shown to be sufficient to give optimal oxygen uptakes and was quite active in the succinic acid dehydrogenase assay (Axelrod, Potter, and Elvehjem, 1942).

RESULTS

I. *Large Doses of Growth Hormone*

In the experiments recorded in Tables 1 and 2, treated animals received daily, for 20 days, 1 mg. of a growth preparation of which 26 gammas, administered daily to hypophysectomized rats 26 to 30 days old, was the

TABLE 1. CHANGES IN BODY WEIGHT, KIDNEY WEIGHT, AND RENAL L-GLUTAMIC ACID DEHYDROGENASE DURING INDUCED GROWTH

Experiment	Average body wt. at start of treatment period in gm.	Average change in body wt. in gm.	Average daily food intake in gm.	Average terminal kidney wt. in gm.	λ Oxygen uptake per hour*			
					Per gm. fresh tissue†	Per organ	Per mg. total nitrogen	Per 100 gm. body wt.†
Series K: Groups IA and IB were fed *ad lib.*, IIA was pair-fed against IA, and IIB against IB. Treatment with growth hormone was started 12 days after operation. Groups IB and IIB received 1 mg. growth preparation per rat daily for 20 days, and were sacrificed the day after the last injection.								
I. Hypophysectomized								
A. Controls	84	−0.6	5.4	0.522	3910 ± 200	2090	137	2440 ± 160
B. Treated	74	72	8.1	0.826	2810 ± 220	2310	99	1580 ± 110
II. Normals								
A. Controls	98	5	5.4	0.771	3000 ± 460	2300	102	2220 ± 290
B. Treated	94	59	8.1	1.012	3340 ± 190	3360	116	2190 ± 230
Series L: Conditions were the same except that groups IB, IIA, and IIB were pair-fed against group IA.								
I. Hypophysectomized								
A. Controls	79	0.5	5.5	0.506	3740 ± 270	1890	112	2370 ± 110
B. Treated	80	30	5.5	0.661	3640 ± 350	2400	117	2170 ± 220
II. Normals								
A. Controls	92	0	5.5	0.702	3480 ± 320	2440	113	2670 ± 200
B. Treated	99	2	5.5	0.787	3160 ± 114	2480	102	2470 ± 180

* Modified Dewan assay; methylene blue used as the carrier.
† Mean ± standard deviation.
Significance of differences (Fisher, 1948): Series K: IA, IB, $P < 0.01$ (per gm. tissue); IA, IB, $P < 0.01$ (per 100 gm. body weight). All rats in each series were of the same age and hypophysectomized ones were 1 month old at operation.

amount required to produce an average gain of 1 gm. per day during 10 days. In the first group (Series K, Tables 1 and 2) the untreated and treated hypophysectomized groups of animals were fed *ad lib.*, and the corresponding normal groups were paired-fed against them. The food consumption of both treated groups (Tables 1 and 2, Series K, groups IB and IIB) was about 50 per cent greater than that of the corresponding controls. The combined effect of this method of feeding, and of the large doses of hormone was to produce striking increases of body weight and kidney weight

TABLE 2. CHANGES IN HEPATIC L-GLUTAMIC ACID DEHYDROGENASE DURING INDUCED GROWTH

Experiment	Average terminal liver wt. in gm.	λ Oxygen uptake per hour*			
		Per gm. fresh tissue†	Per organ	Per mg. total nitrogen	Per 100 gm. body wt.†
Series K: The experimental conditions, initial weights of animals, gains in weight, and food intake are given in Table 1.					
I. Hypophysectomized.					
A. Controls	2.99	2360 ± 580	7,040	68.4	8,430 ± 1930
B. Treated	5.25	1620 ± 270	8,510	52.6	5,810 ± 840
II. Normals.					
A. Controls	2.79	3640 ± 380	10,100	96.8	9,890 ± 1240
B. Treated	4.59	3180 ± 590	14,610	87.4	9,530 ± 1820
Series L: See Table 1 for experimental details.					
I. Hypophysectomized.					
A. Controls	2.90	3300 ± 240	9,960	94.7	12,500 ± 1050
B. Treated	3.33	3540 ± 1510	11,300	96.8	10,400 ± 4040
II. Normals.					
A. Controls	2.45	5460 ± 560	13,400	132.0	14,600 ± 1930
B. Treated	2.84	5400 ± 580	15,300	138.0	15,300 ± 1800

* Modified Dewan assay: Series K, methylene blue; Series L, pyocyanine carrier.
† Mean ± standard deviation.
Significance of differences: Series K, IA, IIA, $P < 0.01$ (per gm. tissue); IA, IB, $P < 0.05 > 0.02$ (per gm. tissue); IA, IIA, $P < 0.2 > 0.1$ (per 100 gm. body weight). Series L, IA, IIA, $P < 0.01$ (per gm. tissue).

(Table 1), and of liver weight (Table 2). In both organs, the amount of glutamic acid dehydrogenase per organ increased in the hypophysectomized treated group, but not nearly so much as did organ weight, so that the concentration of enzyme fell significantly, whether calculated per gm. of fresh tissue, per mg. of total nitrogen, or per 100 gm. of body weight (Series K, group IB, Tables 1 and 2). In the normal treated group (Series K, group IIB, Tables 1 and 2), the increase in enzyme per organ kept pace with organ weight and no significant changes in concentration of renal or hepatic L-glutamic acid dehydrogenase occurred.

In Series L (Tables 1 and 2), all animals received the same amount of food. Moderate gain in weight occurred in the hypophysectomized-treated

group, and virtually none in the normal-treated group (Table 1). Significant changes in the concentration of renal or hepatic L-glutamic acid dehydrogenase did not occur in either group (Series L, groups IB and IIB, Tables 1 and 2).

Results with large doses of growth hormone preparation thus indicate that, in hypophysectomized rats fed *ad lib.*, one can stimulate the formation of kidney and liver tissue in which the concentration of L-glutamic acid dehydrogenase is somewhat low. Deficiency of this enzyme does not, however, account for gain in weight, for it was not observed in normal treated rats fed *ad lib.* (Tables 1 and 2, Series K, group IIB) in which the growth rate was quite rapid, or in hypophysectomized rats in which the growth preparation produced considerable gain in weight despite limited food intake (Tables 1 and 2, Series L, group IB).

It may be noted here that the concentration of hepatic L-glutamic acid dehydrogenase (Table 2) appears to be much lower in the hypophysectomized control groups than in the normal control groups when calculated per gm. of fresh tissue or per mg. of total nitrogen, but that the difference is less significant when the amount of the enzyme in liver per 100 gm. of body weight is considered. This finding is analyzed further in the section in which effects of thyroxin and food intake are considered.

II. *Small Doses of Growth Hormone*

All groups of rats for which data are given in Table 3 were hypophy-

TABLE 3. RENAL AND HEPATIC L-GLUTAMIC ACID DEHYDROGENASE IN HYPOPHYSECTOMIZED RATS GIVEN SMALL DOSES OF GROWTH HORMONE

Daily dose of growth hormone, gamma	Organ	Average terminal organ wt. in gm.	λ Oxygen uptake per hour*			
			Per gm. fresh tissue†	Per organ	Per mg. total nitrogen	Per 100 gm. body wt.†
0	Kidney	0.504	5640 ± 790	2,850	192	3,590 ± 560
20	Kidney	0.566	5610 ± 1400	3,160	185	3,870 ± 1180
40	Kidney	0.547	5610 ± 820	3,060	191	3,740 ± 620
80	Kidney	0.615	4760 ± 680	2,920	164	3,230 ± 620
0	Liver	3.16	9100 ± 1570	28,520	281	35,900 ± 4730
20	Liver	3.00	8630 ± 1060	25,720	252	31,400 ± 3940
40	Liver	3.14	7680 ± 1750	24,000	227	29,400 ± 7160
80	Liver	3.52	8820 ± 1130	30,800	299	33,800 ± 3300

* Assayed according to Copenhaver, McShan, and Meyer (1950). Cytochrome *c* carrier.
† Mean ± standard deviation.

All rats used in this experiment were hypophysectomized when 1 month old, and were fed *ad libitum*. Treatment with growth hormone was begun 17–19 days after operation. Each rat received 10 daily injections of the amount of hormone indicated for its group, and was sacrificed on the day following the last injection.

There were 5 rats per group. The average body weights at the start of treatment in the 4 groups were 80, 75, 69, and 72 gm. respectively, and the average weight gains during treatment were −0.6, 7.8, 13.8, and 19.6 gm.

sectomized and fed *ad lib.* Treated groups received 20, 40, or 80 gammas of the growth preparation daily for 10 days. No significant changes in renal or hepatic L-glutamic acid dehydrogenase concentrations occurred. Thus it is again evident that growth can be induced without diminishing the concentration of this enzyme. Average gains in weight recorded in the footnote of Table 3 bore a linear relationship to the logarithm of the dose (Marx, Simpson, and Evans, 1942). The statement at the start of the preceding section, concerning potency of this growth preparation, is based on this assay.

III. *Effects of Thyroxin and Manner of Feeding*

Administration of 50 gammas of thyroxin daily for 7 days to the treated groups of Series N (Table 4) yielded two results of interest. In the hypophy-

TABLE 4. EFFECTS OF THYROXIN AND MANNER OF FEEDING ON HEPATIC L-GLUTAMIC ACID DEHYDROGENASE

Experiment	Average body wt. at start of treatment period in gm.	Average change in body wt. in gm.	Average daily food intake in gm.	Average terminal liver wt. in gm.	λ Oxygen uptake per hour*			
					Per gm. fresh tissue†	Per organ	Per mg. total nitrogen	Per 100 gm. body wt.†
Series N: Groups IA and IB were fed *ad lib.*; IIA was pair-fed against IA and IIB against IB; groups IB and IIB received 50 gammas of thyroxine per rat daily for 7 days, beginning 28 days after operation, and were sacrificed the day after the last injection.								
I. Hypophysectomized								
A. Controls	73	0.3	4.77	3.01	9,580 ± 1490	27,900	288	38,300 ± 6130
B. Treated	74	0.8	4.66	2.55	10,400 ± 600	28,400	295	38,000 ± 1470
II. Normals								
A. Controls	92	3.2	4.77	2.73	16,900 ± 2290	43,100	441	45,500 ± 6850
B. Treated	93	−15.6	4.66	2.15	16,400 ± 1140	35,300	445	45,300 ± 4860
Series O: Groups IA and IIB were fed *ad lib.*, IIA was pair-fed against IA; no treatment was employed, and all rats were sacrificed at the age of 2 months. Data for body weight and food intake are for the last 20 days prior to sacrifice.								
I. Hypophysectomized								
A. Controls	65	2.0	4.74	2.81	7,560 ± 970	21,200	226	31,600 ± 4600
II. Normals								
A. Controls	86	−2.0	4.74	2.28	12,200 ± 590	27,800	307	33,300 ± 5000
B. Controls	132	65.0	14.0	8.11	8,150 ± 1480	66,400	258	33,000 ± 7000

* Assayed according to Copenhaver, McShan, and Meyer (1950). Cytochrome *c* carrier.
† Mean ± standard deviation.
All rats in each series were of the same age and all hypophysectomized rats were 1 month old at operation.

sectomized rats, group IB, no loss of weight occurred. The normal treated group IIB, which received the same amount of food, lost weight rapidly. Anabolic effects of thyroid preparations in a cretin, in doses which were catabolic in normal subjects, were observed many years ago by Janney (1918). The other result of interest is that the apparently low values for hepatic L-glutamic acid dehydrogenase per gm. of fresh tissue, per mg. of total nitrogen, or per 100 gm. of body weight, are again evident in the hypophysectomized groups of Series N (Table 4) and that thyroxin, like growth hormone (Table 2), did not alter this situation. Experiments on the effect of manner of feeding appear to solve this problem. All groups

in Series O (Table 4) were untreated. Normal group IIA was paired-fed against the hypophysectomized group IA, while normal group IIB was fed *ad lib.* It is evident that the concentration of hepatic L-glutamic acid dehydrogenase in hypophysectomized rats is only low when comparison is made with group IIA. It would seem safe to conclude that this enzyme accumulates in the liver of normal rats in which growth has been arrested by limiting the food intake.

DISCUSSION

Fraenkel-Conrat, Simpson, and Evans (1942) studied the effect of hypophysectomy and of purified pituitary hormones on liver arginase in rats. Growth was stimulated with doses of growth hormone which did not affect the concentrations of liver arginase. Bartlett and Glynn (1950) did find that glutamic-oxalacetic transaminase in muscle, where the principal nitrogen storing effect occurs, was diminished somewhat during growth induced with growth hormone. In liver, the concentration of the enzyme was elevated in the treated animals, and it was suggested that this might be related to increased formation of amino acids from carbohydrate precursors. Whether or not this interpretation is correct, hepatic L-glutamic acid dehydrogenase, which is required along with transaminases in catabolism of amino acids, is in any event not increased during induced growth. Under suitable conditions, it may indeed decrease, but, under other conditions pertaining to the manner of feeding or dosage of hormone, growth can be induced in hypophysectomized and in normal rats without decrease in the concentration of this enzyme.

The observation that the concentrations of hepatic L-glutamic acid dehydrogenase in hypophysectomized and normal groups of rats are similar when both groups are fed *ad lib.*, and quite different when paired feeding is employed, is also of interest. Identical food consumption in two groups of animals whose food requirements are different is no guarantee that conditions are physiologically comparable. In the present experiments, at least, the interpretation appears clearer when both groups of animals are fed *ad lib.*

SUMMARY

In hypophysectomized rats fed *ad libitum*, large doses of growth hormone stimulated formation of liver and kidney tissue having a low content of L-glutamic acid dehydrogenase. A decrease in the concentration of this enzyme in tissue does not, however, appear to be the mechanism through which increases in body weight or organ weights are brought about, for it was not observed in normal animals fed *ad libitum* and treated with large doses of growth hormone, or in hypophysectomized rats fed *ad libitum* and treated with small doses which caused considerable gain in

body weight and organ weights. Thyroxin did not alter the activity of L-glutamic acid dehydrogenase in liver tissue. The concentration of hepatic L-glutamic acid dehydrogenase in hypophysectomized rats was comparable with that of normal ones when both groups were fed *ad libitum*. In normal rats paired-fed against hypophysectomized ones arrest of growth seemed to cause an accumulation of the enzyme in the liver.

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EFFECTS OF FEEDING RAPE OIL ON SOME ENDOCRINE FUNCTIONS OF THE RAT

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IT HAS been shown in earlier experiments that feeding rape oil to rats over a period of a month increases the amount of cholesterol in the adrenals to three to five times that of normal rats, and this effect has been found to be produced mainly by erucic acid, one of the component fatty acids of rape oil (Carroll 1951, 1952).

It seemed of interest to study the functional activity of these adrenals containing large amounts of cholesterol since factors which affect the secretory activity of the adrenal cortex also cause alterations in adrenal cholesterol, and there is suggestive evidence that cholesterol may serve as a precursor of adrenal cortical hormones (Sayers, 1950). In the earlier experiments however, there was little evidence of altered adrenal function as a result of the accumulation of cholesterol, and rats have been fed on a fox chow diet containing 25% by weight of rape oil for as long as eighteen months without apparent effects other than the increase in adrenal cholesterol content. Further studies on this problem have been in the nature of provocative tests designed to accentuate potential differences in the ability of adrenals containing such large amounts of cholesterol to produce cortical hormones. This paper is a report of the results of these experiments.

Studies on female rats have shown that feeding rape oil increased the concentration of cholesterol in the ovaries as well as the adrenals, and a few experiments are reported relating to ovarian function.

EXPERIMENTAL

Young male rats of the Sprague-Dawley strain, weighing 80–90 gm. at the start of the experiments, were used except where otherwise indicated. Details of the diets used are given in connection with the individual experiments. Blood sugars were determined by the method of Shaffer and Somogyi (1933), liver glycogens by the method of Good, Kramer and Somogyi (1933), and urinary nitrogen by the micro-Kjeldahl method.

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Adrenal ascorbic acid and cholesterol were measured as reported previously (Carroll, 1951). Blood eosinophils were measured as described by Randolph (1944).

RESULTS

The known actions of adrenal hormones as well as the physiological and biochemical changes resulting from deficiencies or excesses of these hormones have been reviewed by Noble (1950). The following experiments were designed to show whether rats fed on rape oil differed from control rats in any way that could be correlated with altered adrenal cortical function.

For each test two groups of male rats were used, one group being fed on fox chow and the other on fox chow to which 25% by weight of rape oil[2] was added. Unless otherwise stated, the diets were fed from four to eight weeks before the tests were performed.

(a) *Effect of fasting*

In this experiment two groups of six rats each were subjected to a 48 hour fast during which time their blood sugars and urinary nitrogen excretion were measured. They were then killed and analyses were made for liver glycogen and adrenal cholesterol. The results are shown in Figure 1 and Table 1. The group which had been fed a rape oil diet prior to the

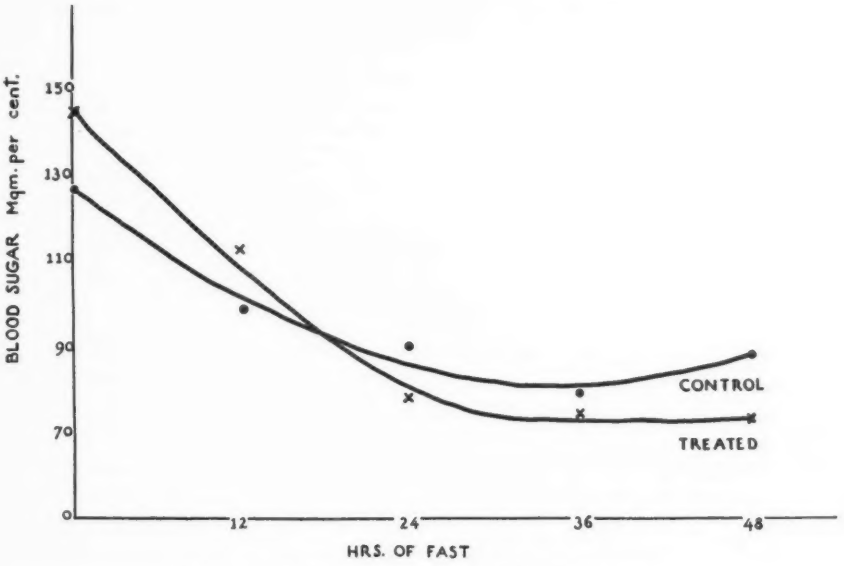

FIG. 1. Effect of fasting following a rape oil diet.

TABLE 1. EFFECT OF FASTING FOLLOWING A RAPE OIL DIET

	Number of rats	Urinary nitrogen, mgm./100 gm. body wt./48 hr.	Liver glycogen, mgm./100 mgm. liver wt.	Adrenal cholesterol, mgm./100 mgm. adrenal wt.
Fox chow diet	6	123.5	0.51 ± 0.07	5.5 ± 0.59
Rape oil diet	6	110.5	0.14 ± 0.06	13.2 ± 0.80

Results are given as average value ± standard error.

period of fasting excreted less urinary nitrogen than the control group. Their liver glycogen was lower and their blood sugar showed no elevation towards the end of the fast. The deviations from normal are, however, not very marked and these results indicate that the rats on rape oil have essentially normal or possibly slight hypofunction of the adrenal cortex.

(b) *Insulin sensitivity*

Adrenalectomized rats have been shown to be much more sensitive to insulin injections than normal rats (Swann and Fitzgerald, 1938). In the present experiments control and rape oil fed rats were fasted for 18 hours and then injected intraperitoneally with 2–4 units of crystalline zinc-insulin (Insulin-Toronto 20 units /cc.) per kilogram of body weight. The insulin was diluted with saline so that the volume injected was approximately 1 cc. On the basis of this test, rats fed on rape oil showed no evidence of adrenal insufficiency since from the results shown in Table 2 they were at least as resistant to insulin injection as control rats.

TABLE 2. INSULIN SENSITIVITY IN FASTED RATS[1]

		Controls	Rape oil fed
Dosage of	2	4/6	1/3
Insulin	3	6/6	1/9
(mgm./kgm.)	4		3/6

[1] Results are given as the proportion of rats which convulsed following any given dosage of insulin.

(c) *Diuresis following a water load*

For this test, six male rats were fed a diet containing 25% rape oil for six weeks. Then together with a control group of six rats, they were fasted overnight, but were given drinking water ad lib. The following morning they were given water amounting to 5% of their body weight by stomach tube as in the Burn antidiuretic assay (Gilman and Goodman, 1937). The time required to excrete 50 per cent of this water load was 110 minutes in the treated as compared with 95 minutes in the control rats. Adrenalectomized rats excrete a water load more slowly than normals (Gaunt, 1946),

but differences of the order observed in this experiment are not considered to be significant.

(d) *Exposure to cold*

Young male rats on a diet containing 25% by weight of rape oil failed to survive continued exposure to temperatures of 0–3 C. In a typical experiment, a group of six rats averaging 108 gm. body weight were put on a 25% rape oil diet. They were kept at room temperature for 3 days and then placed in a cold room at 0–3 C. and maintained on the same diet. Growth ceased as soon as the animals were exposed to the cold, and, after a period of several days to a week, the tails and hind feet became very red, and the hind feed were sometimes swollen and edematous. In some rats, the tips of the tails became gangrenous and either dropped off or were chewed off by other animals in the group. The rats died after one to three weeks in the cold room. In younger rats, death occurred in a shorter time while older rats survived much better, and, in some cases, survived for more than two months and were still in good condition at the end of the experiment.

The effects which have been described are probably due in large part to the high fat diet. Feeding a diet containing 25% by weight of sesame oil produced similar effects, although this oil has little effect on adrenal cholesterol (Carroll, 1951). On the other hand, rats which were fed on diets containing 25% of olive oil or of margarine generally survived exposure to cold, although their growth rates were suppressed. Rats on fox chow alone remained healthy and continued to grow. The results were undoubtedly also influenced by the condition of the rats' fur since this furnishes insulation for the animals. Thus, rats on rape oil or sesame oil tend to become greasy after some days on the diet while this is generally not true of rats fed margarine or olive oil.

Several attempts were made to influence the survival time of rats on rape oil diets in the cold by administration of cortisone acetate, desoxycorticosterone acetate, adrenocorticotrophic hormone, saline pituitary extracts or by unilateral adrenalectomy. These experiments did not give any indication that the observed effects were related to abnormal adrenal function.

(e) *Response of adrenal ascorbic acid and cholesterol to removal of one adrenal*[3]

The removal of one adrenal from normal rats was found to produce a marked depletion in the concentration of ascorbic acid and cholesterol of

[3] This experiment was performed by Mr. J. M. Salter.

the second adrenal, an effect similar to that resulting from stress or the administration of ACTH (Sayers and Sayers, 1948).

In order to compare the response in rats fed on rape oil to that of normal rats, two experiments were performed. Groups of six control and six rape oil fed rats were anesthetized with nembutal and one adrenal was removed. Then one hour later in the groups used for ascorbic acid determinations,

TABLE 3. CHANGES IN ADRENAL ASCORBIC ACID AND CHOLESTEROL IN RESPONSE TO REMOVAL OF ONE ADRENAL

		Fox chow diet	Rape oil diet
	A—Ascorbic Acid		
	Rat Body Weight	199	169
Adrenal ascorbic acid (mgm./100 gm. adrenal weight)	first adrenal	444	408
	second adrenal	217	209
	difference	227 ± 15	199 ± 9.5
Absolute difference in ascorbic acid in the two adrenals (mgm.)		0.0273 ± 0.0012	0.0315 ± 0.0017
	B—Cholesterol		
	Rat Body Weight	199	161
Adrenal cholesterol (mgm./100 mgm. adrenal weight)	first adrenal	4.0	10.3
	second adrenal	2.1	8.5
	difference	1.9 ± 0.43	1.8 ± 0.38
Absolute difference in cholesterol the two adrenals (mgm.)		0.240 ± 0.018	0.181 ± 0.014

and four hours later in the groups used for cholesterol determinations, the second adrenal was removed. The levels of ascorbic acid and cholesterol were compared in the two adrenals of both treated and control groups with the results shown in Table 3. It can be seen that the lowering of ascorbic acid and cholesterol is much the same in both groups in spite of the marked difference in cholesterol content between the treated and control groups.

Even the injection of large doses (50 mgm.) of ACTH intraperitoneally failed to reduce the concentration of adrenal cholesterol to very low levels in rats fed on rape oil. Four hours after such injection two rats had 9.6 and 8.4% of cholesterol in their adrenals while three other untreated rats from the same group had 14.6, 12.9 and 12.1% of cholesterol respectively in their adrenals.

RELATION OF ADRENAL HYPERTROPHY PRODUCED BY RAPE OIL TO CHANGES IN ADRENAL SIZE AND COMPOSITION PRODUCED BY OTHER MEANS

The experiments described in this section constitute another approach to the problem, in which rape oil feeding was combined with other treatments known to affect adrenal size and composition (Tepperman, Engel

TABLE 4. EFFECT OF VARIOUS TREATMENTS WITH AND WITHOUT RAPE OIL FEEDING ON THYMUS AND ADRENAL WEIGHTS

	ACTH[1]		Thyroid		High protein[2]		Tumor Bearers[3]	
	0% rape oil	25% rape oil	0% rape oil	25% rape oil	0% rape oil	25% rape oil	0% rape oil	25% rape oil
Number of rats	6	6	5[4]	5	4	5	6	6[5]
Days on diet	27	27	30	30	27	27	62	62
Body weight[6] (gm.)	164 ±14	137 ±7.7	154	135	236 ±11	222 ±5.5	316 ±9.7	239 ±26
Thymus weight (mgm.)	55 ±21	34 ±6.0	332	166	615 ±57	575 ±38	—	—
Adrenal weight (mgm.)	72.0 ±5.3	69.5 ±4.5	51.4	58.4	39.9 ±4.0	52.3 ±4.3	43.3 ±2.8	63.4 ±2.9
Total adrenal cholesterol (mgm./100 mgm. of adrenal weight)	4.6 ±0.54	6.4 ±0.45	3.9	10.7	4.9 ±0.61	10.0 ±0.54	3.5 ±0.63	6.6 ±1.6

	Stilbestrol		Cortisone		Controls	
	0% rape oil	25% rape oil	0% rape oil	25% rape oil	0% rape oil	25% rape oil
Number of rats	5	5	6	6	24	24
Days on diet	31	31	27	27	28	28
Body weight[6] (gm.)	105 ±4.2	85 ±1.0	199 ±4.8	140 ±10.3	189 ±3.1	157 ±3.6
Thymus weight (mgm.)	463 ±65	276 ±17	329 ±23	142 ±27	417 ±23	303 ±21
Adrenal weight (mgm.)	29.9 ±1.1	30.0 ±1.4	24.1 ±0.76	20.3 ±0.98	30.2 ±0.41	37.5 ±0.81
Total cholesterol cholesterol (mgm./100 mgm. of adrenal weight)	1.5 ±0.09	8.5 ±0.74	2.4 ±0.24	10.5 ±0.58	4.4 ±0.28	12.0 ±0.65

[1] In most of these experiments, one group of rats was fed fox chow and the other group fox chow to which 25% by weight of rape oil was added. In those involving ACTH, a synthetic died was used, consisting of 25% by weight of casein, 45% glucose, 20% lard, 5% corn oil, 5% of salt mixture and a complete supplement of vitamins. In the alternate diet, 25% rape oil was substituted for the lard and corn oil. The control values are for the fox chow diets but similar control values were obtained with these synthetic diets.

[2] The high protein diet consisted of 70% by weight of casein, 20% lard, 5% corn oil, 5% salt mixture and a complete vitamin supplement. In the alternate diet, 25% rape oil was substituted for the lard and corn oil.

[3] Each rat was injected subcutaneously with a suspension of cells from a malignant fibrosarcoma on the 34th day of this experiment. This tumor was originally induced by 1,2-dimethyl-9,10-benzanthracene and had been maintained through thirteen generations by repeated transplantation.

[4] Three rats in this group died during the experiment.

[5] One rat in this group died during the experiment.

[6] The initial starting weight of the different groups of rats varied from 77 to 90 gms.

Results are given as average value ± standard error.

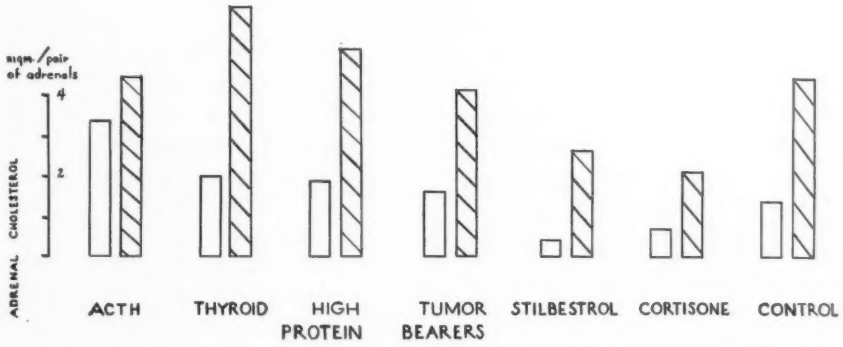

FIG. 2. Effect of various treatments with and without oil feedings, on adrenal cholesterol. The open bars represent groups not receiving rape oil while the hatched bars represent groups fed rape oil.

and Long, 1943). It was hoped that some of these experiments might accentuate the effects produced on the adrenal by rape oil so that physiological differences might be more apparent. The results are summarized in Table 4 and Figure 2.

(a) *ACTH administration*

When ACTH is given in repeated doses, it causes an increase both in adrenal size and cholesterol content (Sayers, Sayers, Fry, White and Long, 1944). In the present experiment, the rats were kept on diet for four weeks, and 20 mgm. of ACTH (Armour—potency 50% of Armour Standard LA-I-A as measured by the adrenal ascorbic acid depletion test) was injected daily subcutaneously in two equal doses for the last 9 days into each rat. Although the ACTH caused a pronounced increase in adrenal size in both the control and rape oil fed groups, it tended to equalize the cholesterol concentrations, and thus obscure the effect of rape oil (Figure 2).

(b) *Desiccated thyroid feeding*

Adrenal hypertrophy may also result from feeding thyroid preparations (Pekkarinen, Koivusalo and Pyroala, 1951), and, in our experiments, the presence of 1 per cent of dried thyroid (Burroughs, Wellcome & Co.) in the diet caused a large increase in adrenal weight in both the control and rape oil groups. In this case, unlike the experiment with ACTH, the adrenal cholesterol remained much higher in the group on rape oil. Thyroid powder fed at this level was quite toxic to the control group and three of the five rats died during the experiment, whereas none of the rape oil group died. A protective effect of high fat diets in rats fed on thyroid has been described by Greenberg and Deuel (1950).

(c) *High protein diet*

Reference to Table 4 shows that the high protein, high fat diets used in the present experiments caused a moderate increase in adrenal size, but the concentration of adrenal cholesterol appeared to be little affected with the result that the absolute amount of adrenal cholesterol was increased in both groups. These diets gave excellent growth in both the control and the rape oil groups.

(d) *Tumor growth*

The presence of a malignant tumor is also known to cause definite adrenal enlargement, and this result has been confirmed in our experiments. Tumors grew in all animals of both the control and the rape oil fed rats, and the rate of growth did not appear to be significantly different in the two groups. The group on rape oil seemed to be in poorer condition and

one of this group died near the end of the experiment. The adrenal cholesterol in the rape oil group was proportionately higher in this experiment.

(e) *Stilbestrol administration*

Stilbestrol has been reported to cause adrenal hypertrophy with a decrease in the concentration of cholesterol (Skelton, Fortier and Selye, 1949), but in the present experiments where stilbestrol was fed at the level of 2 mgm./100 gm. of diet, adrenal hypertrophy was not prominent. There was however, a proportionate lowering of cholesterol concentration in both the control and rape oil groups, and there was a marked inhibition of growth in both groups.

(f) *Cortisone administration*

In contrast to the treatments which have been described above, the administration of cortisone causes a decrease in adrenal weight, and this decrease is associated with a lowered cholesterol concentration (Winter, Silber and Stoerk, 1950). In our experiments in which 1 mgm. of cortisone acetate (Merck) was injected daily subcutaneously for 27 days to rats on control and rape oil diets, the decrease in adrenal weight was observed in both groups, but the concentration of adrenal cholesterol remained high in the rape oil group.

EFFECT OF RAPE OIL DIETS ON THE OVARY

Kennedy and Purves (1941) reported that female rats fed on rape seed diets matured sexually much later than normal females, in that they would not become pregnant until five to seven months of age. Since preliminary experiments indicated that the ovaries of rats fed on rape oil contained more cholesterol than those of controls, two further experiments were performed to investigate ovarian function in female rats fed a diet containing 25% rape oil.

In the first of these, immature rats were used and the rape oil diet was continued through puberty until the rats reached maturity. As observed in previous experiments, the rats on the rape oil diet grew less well than the controls, and their vaginas opened one to two weeks later than the controls, but at the same approximate body weight. Once the vaginas opened, the sex cycles were followed by taking daily vaginal smears, and no irregularities in the cycles were observed. At the end of the feeding period, the concentration of ovarian cholesterol in the treated group was significantly higher than that in the controls. The rats were killed on days in which their smears showed diestrus.

The second experiment was performed on adult females. Again, no irregularities were noted in the sex cycles, but the concentration of ovarian

cholesterol was significantly higher in the treated group. In both experiments the ovaries were smaller in the treated group with the result that the total amount of ovarian cholesterol was not significantly greater than that of control animals. The results are given in Table 5.

TABLE 5. EFFECT OF RAPE OIL ON OVARIAN CHOLESTEROL

	Days on diet	Number of rats	Rat weight		Total adrenal cholesterol (mgm./100 mgm. adrenal weight)	Ovarian weight (mgm.)	Ovarian cholesterol		
							Free	Total	
			Initial (gm.)	Final (gm.)			(mgm./100 mgm. ovarian weight)	(mgm./100 mgm. ovarian weight)	Absolute[1] amount (mgm.)
					(Immature)				
Control		6	49±1.2	194±4.9	4.5±0.28	54.3±4.4	0.31±0.03	1.41±0.17	0.77
								P <0.02	
25% Rape oil	60	6	47±1.0	153±7.1	13.8±0.45	40.3±4.2	0.39±0.02	2.10±0.18	0.85
					(Mature)				
Control		5		199±2.0	4.3±0.34	68.3±5.0	0.28±0.01	1.11±0.07	0.76
								P <0.01	
25% Rape oil	28	6	209±7.8	211±5.0	10.6±0.51	58.9±3.7	0.35±0.01	1.96±0.19	1.15

[1] Absolute amount = percentage of cholesterol × weight of the paired adrenals. Results are given as average value ± standard error.

DISCUSSION

The experiments which have been described give little indication that the shift in concentration of adrenal cholesterol which results from feeding a rape oil diet is associated with altered adrenal function. If there were marked or even moderate hypofunction, one would expect the rats to be more sensitive to fasting, and to insulin injection. It is true that rats fed on rape oil failed to survive exposure to cold temperatures, but in this case, there was evidence that other factors were responsible.

The evidence against hyperfunction is perhaps not as conclusive, but several points may be noted. One finds a decrease in thymus weight with hyperfunction (cf.—the experiment involving ACTH administration—Table 4), but rape oil feeding produced only a moderate decrease in thymus weight. The blood sugar of treated rats was in the normal range and there was no evidence of glycosuria in any cases in which the urine was tested. Blood eosinophils were measured on several occasions and no definite trends were noted in rats fed on rape oil as compared to control rats.

The experiments in which the rape oil diet was combined with other for forms of treatment (Table 4) give little indication of potential defects resulting from feeding rape oil diets. In most of the experiments, the effect of rape oil on adrenal cholesterol seems merely to be superimposed on the effects produced by other agents. A possible exception is the experiment involving ACTH in which the ACTH appeared partially to obscure the effect of the rape oil. This experiment may be contrasted to a previous experiment in which rape oil was fed to rats for a month and then the rats

were hypophysectomized. Although the adrenals atrophied as one would expect when the source of endogenous ACTH was removed, the cholesterol concentration remained at a very high level (Carroll, 1951). The effect of rape oil on adrenal weight is not normally very prominent and this effect was overshadowed by the effects of the other agents.

The thymus weights show interesting variations. Decrease in thymus weight may be related to increased adrenal activity as is well shown in the experiment involving ACTH. However, although the rats on high protein diet have enlarged adrenals, their thymus weights are larger even than those of control animals on a normal diet. These rats grew exceptionally well, and generally speaking, good growth seems to be associated with a large thymus, but it is curious to note that in the groups fed stilbestrol, growth was minimal, but the thymus weights were approximately normal.

Other than the adrenal, the ovary is the only other organ which contains most of its cholesterol in the esterified form and it would be natural to expect that rape oil would cause a similar marked increase in ovarian cholesterol. While such an effect was observed, it was much less striking than the changes in the adrenal and there was no obvious interference with ovarian function.

The earlier reports on feeding rape seed to rats showed pronounced changes in pituitary cell structure, which appeared to be correlated with the anti-thyroid activity of the seed (Griesbach, 1941). With the collaboration of Dr. J. S. Thompson,[4] differential cell counts have been performed on the pituitaries of groups of male and female rats fed on diets containing 25% rape oil for periods of a week to a month. The changes described by Griesbach were not found in these experiments, with the possible exception that there appeared to be a slight increase in basophils. It is, therefore, probable that changes in the pituitary found by the earlier workers are related to the anti-thyroid effects produced by rape seed.

SUMMARY

The accumulation of adrenal cholesterol which results from feeding rape oil to rats appears not to be associated with marked changes in adrenal function, and such adrenals respond normally to stimulation. When rape oil feeding is combined with other regimes known to affect adrenal size and cholesterol content, the effects of the combined treatments seem to be additive.

Rape oil diets cause a similar though less marked accumulation of ovarian cholesterol, but no interference with ovarian function was observed.

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THE WORK PERFORMANCE OF NORMAL RATS GIVEN CONTINUOUS INTRAVENOUS INJECTIONS OF CORTISONE AND OF CORTICOTROPIN

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DURING the past 20 years there have been a number of reports that normal animals can be made more resistant to fatigue by the administration of adrenal cortical extracts. One of us (DJI) has made several attempts to confirm these claims with negative results. One of our studies (Ingle, Nezamis and Jeffries, 1949) has been published. The present report is based upon positive results obtained by the continuous intravenous injection of either cortisone or corticotropin.

METHODS

Male rats of the Sprague-Dawley strain were obtained from the Upjohn breeding colony and maintained on Archer Dog Pellets until they reached a weight of 200 ± 2 gms. The work test was carried out according to Ingle (1944). The animals were anesthetized with phenobarbital sodium and cyclopal sodium and were subjected to the stimulation of muscle immediately following hypophysectomy. The gastrocnemius muscle of the left hind leg was weighted with 100 gms. A Nerve Stimulator, Model B (Upjohn) was used to deliver 5 pulses per second. The duration of each pulse was 20 milliseconds and the intensity was 20 milliamperes. The distance the weight was lifted was recorded on automatic work adders. Each recorder revolution represented approximately 400 gram-centimeters of work. Stimulation was continued for 48 hours. The animals were enclosed in a cabinet with the temperature constant at $28 \pm 0.5°$ C.

Non-esterified cortisone was used in these experiments. It was made up in a solution of saline with 5 per cent ethanol. The controls of the cortisone treated rats received equal volumes of physiological saline containing 5 per cent ethanol. The corticotropin (Upjohn) was prepared from the pituitary glands of domestic packing house animals and was described as being practically free of other biologic principles. Its potency is expressed in terms of U.S.P. units as determined by the ascorbic acid depletion test (Sayers, Sayers and Woodbury, 1948). This preparation is stable at room temperature for several days. The corticotropin was added to physiological saline. The controls for these animals treated with corticotropin received equal volumes of physiological saline.

The fluid load was 20 cc. per 24 hours per rat. The injections were made into the jugular vein by means of a continuous injection apparatus which was incorporated into the fatigue machine. Each position on the 12-place apparatus and each of the 12 work recorders was represented equally among the control and the experimental animals.

The glucose content of tail blood was determined by the method of Miller and Van Slyke (1936).

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EXPERIMENTS AND RESULTS

In experiment 1 (Table 1) 57 untreated rats served as controls for an equal number which were grouped according to the amounts of corticotropin per 24 hours per rat: 24 rats, 5 units; 18 rats, 10 units; and 15 rats, 20 units. In each group the rats which were treated with corticotropin performed a greater average amount of work than did the untreated animals. The differences between the averages for treated and untreated rats within any one group were not statistically significant but when the values for the three groups are pooled together the direction of the difference is highly significant.

TABLE 1. AVERAGE TOTAL AMOUNTS OF WORK BY NORMAL RATS

Experiment	Test substance	Daily dose	Number pairs	Means and standard deviations		Difference
				Treated	Untreated	
1	corticotropin	5 μ	24	101,139 ± 2,810	91,777 ± 2,825	9,362 ± 3,984
		10 μ	18	101,424 ± 2,074	93,952 ± 3,490	7,472 ± 4,060
		20 μ	15	101,209 ± 3,723	94,701 ± 4,360	6,508 ± 5,734
2	cortisone	4 mgs.	19	112,977 ± 2,895	90,968 ± 4,035	22,009 ± 3,829
		6 mgs.	20	109,339 ± 3,047	90,265 ± 3,065	19,074 ± 4,322
3	cortisone	4 mgs.	12	107,290 ± 3,834	88,058 ± 5,670	19,232 ± 6,844

In experiment 2 (Table 1) 39 rats served as controls for an equal number which were grouped according to the following amounts of cortisone per 24 hours per rat: 19 rats, 4 mg. and 20 rats, 6 mg. In each of the two groups the rats treated with cortisone performed a significantly greater average amount of work than did their untreated controls.

In experiment 3 (Table 1, Figure 1), 12 rats received 4 mg. of cortisone daily and an equal number of animals served as untreated controls. The level of blood glucose and the rate of work for each rat was determined after 6, 24 and 48 hours of work. The average level of blood glucose as well as the average rate of work remained higher in the rats treated with cortisone than in the untreated controls.

DISCUSSION

The finding (experiment 3) that the higher than normal average rate of work by the rats treated with cortisone was correlated with a higher than normal average blood glucose value is one basis for the hypothesis that an increased rate of gluconeogenesis from protein may have provided extra carbohydrate to the contracting muscle. There is no direct evidence from, these experiments as to the metabolic basis for the positive effects of cortisone and of corticotropin upon work output. It is known from other studies that large doses of cortisone do stimulate gluconeogenesis (Welt *et al.*, 1952) and that the administration of exogenous glucose does im-

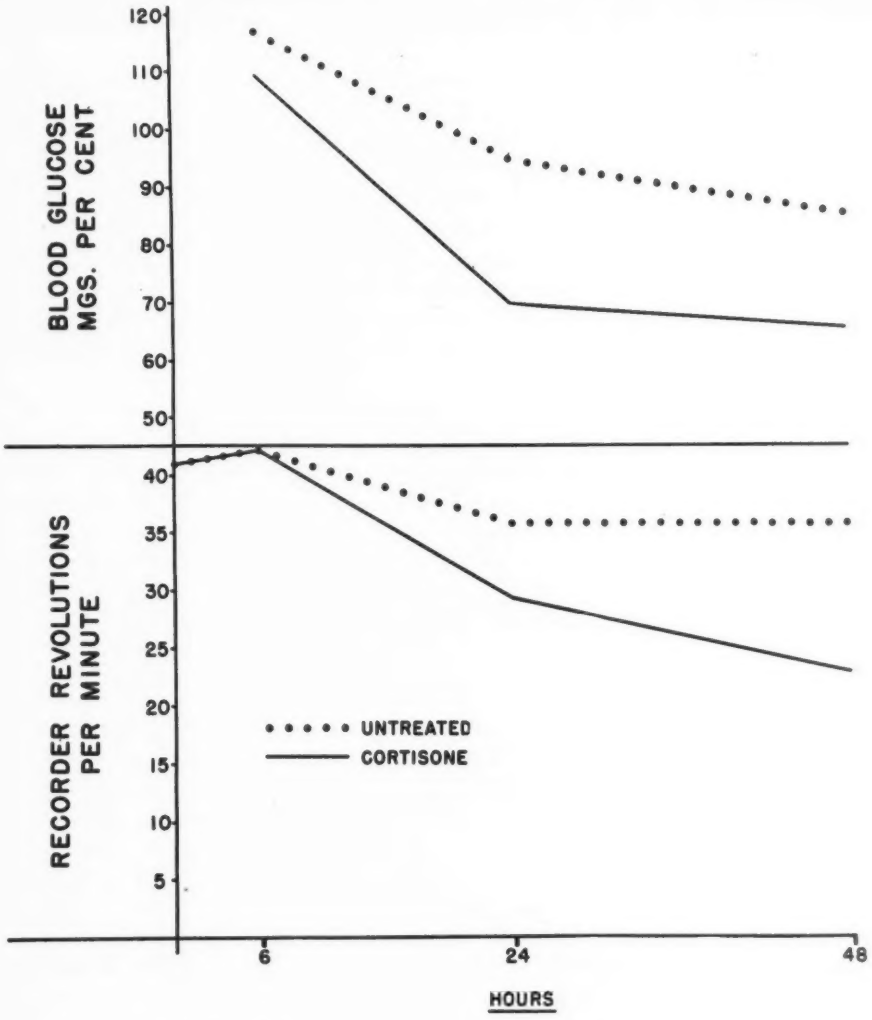

FIG. 1. Correlation between rates of work and the level of blood glucose in normal rats. Averages for 12 rats per group.

prove the performance of muscle under similar conditions of experiment (Ingle, Nezamis and Morley, 1950).

These results agree with the findings of del Pozo *et al.* (in press), who have shown that the intravascular administration of cortisone hemisuc-

cinate to the normal cat causes enhancement of striate muscle response to faradic stimulation.

Other studies from this laboratory (Ingle, Nezamis and Morley, 1952) have shown that the requirement for adrenal cortical hormones by the adrenalectomized rat is greatly increased by the stress of muscle work. When the normal rat is subjected to the prolonged faradic stimulation of muscle the adrenal cortices undergo rapid and extensive hyperplasia during the time that the other tissues of the animal are being rapidly depleted (Ingle, 1938). Our earlier failures to demonstrate a positive effect of the adrenal cortical hormones upon the work output of normal rats (Ingle, Nezamis and Jeffries, 1949) may have been due to inadequate dosage. The amounts of corticotrophin and of cortisone used in the present studies were very large.

There have been many claims that these hormones are useful in relieving the muscular asthenia of patients who do not have recognizable diseases of either the anterior hypophysis or of the adrenal cortex. There are also word-of-mouth reports that the administration of these hormones to athletes and to dogs and horses used in racing causes improved muscular performance. Proof for these claims is lacking but further studies of the effect of these hormones upon muscular performance in patients, normal human subjects and animals are indicated.

SUMMARY

Normal male rats of 200 gms. weight were anesthetized with barbiturates and subjected to faradic stimulation of the gastrocnemius muscle to lift a 100 gm. weight 5 times per second. Stimulation was continued for 48 hours. Either corticotropin or cortisone was administered by continuous intravenous injection. One rat of each pair received an equal fluid load (20 cc. per 24 hours) without hormone. The work output of the rats treated with corticotropin (5, 10 and 20 units per rat per day) was increased above the normal average by about 8.6 per cent. The work output of the rats treated with cortisone (4 and 6 mg. per rat per day) was increased above the normal average by about 22.4 per cent.

Acknowledgement

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THE BIOASSAY OF PITRESSIN AND ANTIDIURETIC SUBSTANCES IN BLOOD AND URINE[1]

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ALTHOUGH many methods have been proposed for the assay of the antidiuretic hormone of the neurohypophysis, a simple, precise procedure is still not available (vanDyke, 1952). The present report is concerned with a procedure which meets the requirements for simplicity and the precision essential for relatively accurate bioassays. The method is based on the observation that when rats are given a gavage of saline which is repeated one hour later, the volume of urine excreted after the second gavage is essentially linear on time for sixty minutes. Accordingly, the volume excreted during an early portion of the sixty minute interval bears a constant relation to that excreted at the end of that interval so that the ratio of the former to the latter can be utilized as an expression of the diuresis.

METHOD

Male albino rats, weighing from 140 to 225 grams, were used after a preliminary fast of approximately 16 hours during which time the animals were permitted access to water. All assays were performed in a room maintained at 27°–29° C. Five ml. of warm 0.2% NaCl per 100 grams body weight were administered by stomach tube. One hour later, the gavage was repeated and at twenty minute intervals, thereafter, the volume of urine excreted was measured. The animals were handled and prodded just prior to each measurement of urine volume in order to ensure the emptying of their bladders as completely as possible. Twenty minutes after the second gavage, 1 ml. of isotonic saline per 100 grams body weight or a similar volume of a solution of an antidiuretic substance (ADS) in isotonic saline was injected intraperitoneally. In some instances, the volume of urine excreted at the end of the first hour after the first gavage was measured also.

After establishing the manner of expressing the response to an antidiuretic agent, the relationship of the response to the dose was determined.

For the assay of the antidiuretic substances, the experimental design of Bliss (1944) was employed. Four equally sized groups of rats were selected at random and Pitressin was assayed against itself. Two groups were injected with a high and a low dose of a standard preparation of Pitressin[3] and the other two groups with a high and a low dose

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[2] U. S. Public Health Service Research Fellow of the National Institute of Mental Health.

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of an "unknown" quantity of that preparation. The concentrations of both the standard and the "unknown" were chosen so that the ratio of the high to the low dose of the standard was the same as the ratio of the high to the low dose of the "unknown." The same design was utilized when blood plasma or urine was assayed for antidiuretic activity. When urine was employed, an extract was prepared by the procedure described by Grollman and Woods (1949).

RESULTS

After rats are given two gavages of 5 ml. of 0.2% NaCl per 100 grams body weight, the excretion of urine after the second gavage is essentially

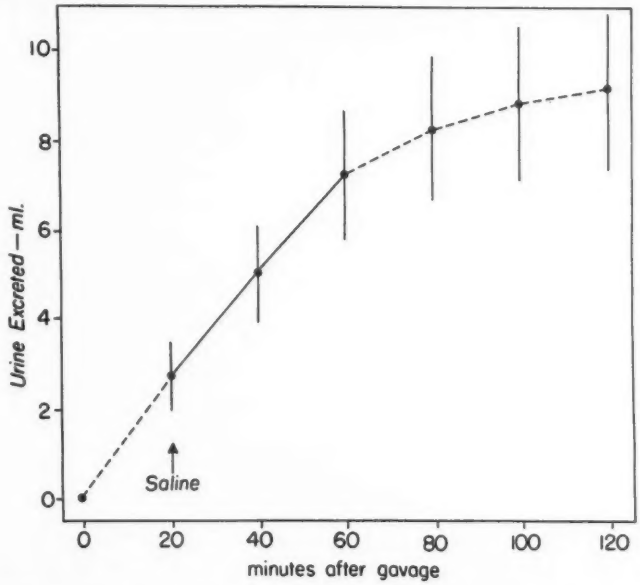

FIG. 1. Mean of Diuresis Curves of 60 Rats. One hour prior to test period, 5 ml. 0.2% NaCl per 100 gm. body weight were administered by gavage. A similar quantity was given at 0. At 20 minutes, 1 ml. isotonic saline per 100 gm. body weight was injected intraperitoneally. Vertical lines illustrate the S. D. of the mean volume excreted by each interval.

linear on time for sixty minutes. Figure 1 illustrates the mean diuresis curve of 60 rats given an intraperitoneal injection of isotonic saline twenty minutes after the second gavage. A probit plot of the volumes excreted for each time interval reveals that the values distribute themselves normally about the means depicted in Figure 1. It is evident that the segment of the curve between 20 and 60 minutes is essentially linear; the regression coefficient of the volume excreted on time is 0.1143. ($P < 0.001$).

Since the volume excreted from 20 to 60 minutes after the second gavage

is linear on time when an intraperitoneal injection of isotonic saline is given at 20 minutes, it is evident that the volume excreted in 20 minutes bears a constant relation to that excreted in 60 minutes. Thus, for the data illustrated in Figure 1, the ratio of the volume at 60 minutes (V60) to the volume at 20 minutes (V20) = 2.64. The injection of an antidiuretic agent instead of isotonic saline, however, results in a reduction of V60/V20 since the antidiuretic activity produces a diminution in the volume of

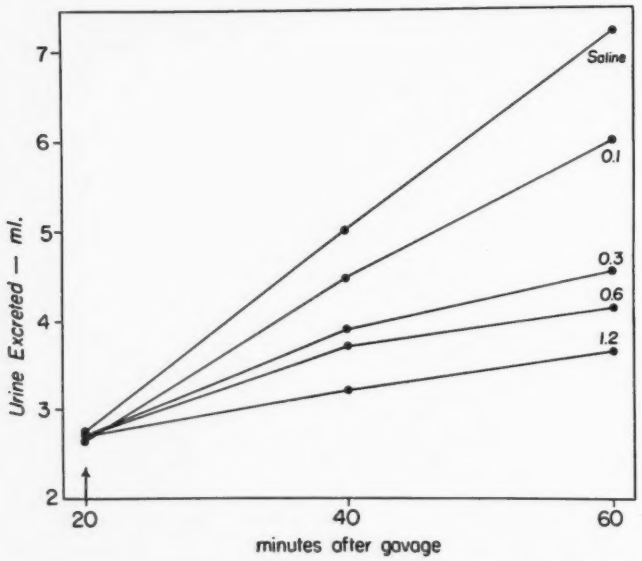

FIG. 2. Influence of Pitressin on Diuresis. One ml. isotonic saline or 1 ml. Pitressin per 100 grams body weight was administered at 20 minutes after the second gavage. Each curve represents the mean of curves obtained with 60 rats.

urine excreted in the interval between 20 and 60 minutes (Figure 2). The reduction of V60/V20 is dependent upon the quantity of antidiuretic agent that is administered since the injection of Pitressin in amounts ranging from 0.1 to 1.2 mU per 100 grams body weight results in a progressive diminution in the volume excreted by 60 minutes (Figure 2). Consequently, the aforementioned ratio can be utilized as an expression of the antidiuretic activity of a substance administered under the conditions described herein.

In order to determine the relation of the magnitude of the response to the dosage of the antidiuretic agent, it was first established that the responses of individual rats to varying doses of Pitressin distributed themselves normally about the means. The best fit between the response and

the dosage was found to be in terms of the reciprocal of the ratio: V60/V20 to the logarithm of the dosage (Figure 3). Thus, the linear regression coefficient of the reciprocal of V60/V20 on the dosage of Pitressin injected was 0.2684 ($P < 0.001$).

Although the volume of fluid administered by stomach tube was based on the weight of the animal, it was essential to establish the relation of the weight of the animal in the range used to the volume of urine excreted.

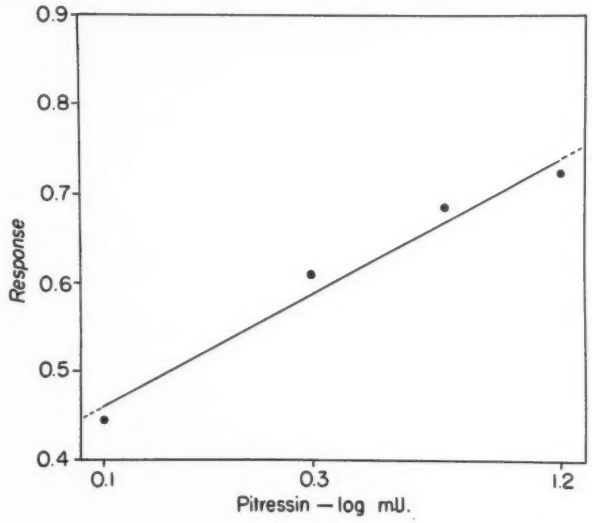

FIG. 3. Relation Between Dose of Pitressin and Antidiuretic Response. The assays were performed as described in the text. Response is expressed as reciprocal of $\frac{\text{urine volume at 60 minutes}}{\text{urine volume at 20 minutes}}$. $b = 0.2684$, $P < 0.001$.

Calculation of the linear regression coefficient of weight on volume excreted at twenty minutes after the second gavage was 0.0101 which was insignificant ($P > 0.2$).

Having established that the response as expressed by the reciprocal of V60/V20 can be used as a measure of the antidiuretic activity of ADS, it became pertinent to compare it with expressions utilized by others as a measure of response. Accordingly, a group of rats was treated in the standard manner and given different quantities of Pitressin by intraperitoneal injection. The response to the injection was then expressed in a variety of ways (Table 1).

The most common response employed for the assay of ADS utilizes the time necessary to attain an excretion of 50% of the volume of fluid (Ralli,

1950). Another expression of the response to an antidiuretic agent is that which relates the volume excreted after the injection of the agent to the volume of the administered fluid that is retained (Birnie, *et al.*, 1950). This response may be expressed as $1-\frac{\text{volume at 80 minutes after second gavage}}{\text{load}}$ $\times 100$, where the load represents the difference between the total volume

TABLE 1. COMPARISON OF VARIOUS EXPRESSIONS OF THE ANTIDIURETIC RESPONSE TO PITRESSIN

Expression of response	mU Pitressin	No. of rats	Mean response ± S.D.	Coefficient of variation %
Minutes required for excretion of 50% of second gavage	0.1	30	34.60 ± 10.55	30.49
	0.3	30	44.73 ± 11.86	26.51
	0.9	29	61.72 ± 28.81	46.68
$1-\frac{\text{vol. 80 min.}}{\text{load*}}\times 100$	0.1	30	38.86 ± 18.79	48.30
	0.3	30	62.63 ± 13.12	20.94
	0.9	29	77.58 ± 9.75	12.57
$1/\frac{\text{urine vol. at 60 min.}}{\text{urine vol. at 20 min.}}$	0.1	30	0.393 ± 0.059	15.01
	0.3	30	0.490 ± 0.089	18.16
	0.9	29	0.634 ± 0.121	19.08

* Load = total volume of fluid administered − total volume of urine excreted by 20 minutes after the second gavage.

of fluid administered and the total volume of urine excreted by 20 minutes after the second gavage.

The linear regression coefficients of the above expressions of the response to the log of the dose of ADS employed were all highly significant. Comparison of the coefficients of variation of the various responses, however, revealed that the expression proposed herein gives the smallest variability (Table 1).

Since it has been claimed that a rat cannot be used too frequently for the assay of an antidiuretic substance, it was essential to determine the effect of repetitive tests. Table 2 summarizes the data obtained when the standard procedure was applied to the same animals every second day for four trials. It is apparent that there was no significant difference between the four trials. Accordingly, it is permissible to use a rat every second day for at least four assays.

Although the administration of ADS by intravenous injection is undoubtedly the most efficient procedure, it is not as convenient as either the intraperitoneal or subcutaneous injection. When Pitressin was administered subcutaneously, however, the regression coefficient of the reciprocal of V60/V20 on the log of the dose was not significant ($b = 0.1115$,

TABLE 2. DIURETIC RESPONSE OF RATS WHEN TESTED EVERY SECOND DAY

Animal	Trial				
	1	2	3	4	Mean
1	0.429	0.308	0.300	0.364	0.350
2	0.417	0.333	0.333	0.300	0.346
3	0.400	0.214	0.400	0.286	0.325
4	0.467	0.400	0.455	0.300	0.406
5	0.385	0.385	0.400	0.583	0.438
6	0.273	0.417	0.400	0.400	0.373
7	0.357	0.375	0.429	0.333	0.374
8	0.455	0.333	0.385	0.357	0.383
9	0.385	0.400	0.333	0.250	0.342
Mean	0.396	0.352	0.382	0.353	0.371

Response expressed as reciprocal of $\frac{\text{urine volume at 60 minutes}}{\text{urine volume at 20 minutes}}$.

Source of variation	Degrees of freedom	Sum of squares	Mean square
Total	35	0.0988	—
Trials	3	0.0096	0.0032
Rats Within Trials	32	0.0892	0.0028

$F = 1.14$, $P > 0.2$

TABLE 3. ASSAY OF THE ANTIDIURETIC ACTION OF PITRESSIN

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}}$$

(Unknown = Standard)

N*	Dosage levels, mU	Relationship: log dose—Response			
		b	λ	t†	Potency ratio ±S.E.
5	0.10–0.30	0.2752	0.2081	0.996	106.30 ± 22.79
5	0.10–0.30	0.2341	0.2465	0.926	75.36 ± 19.74
5	0.10–0.30	0.1367	0.6153	1.165	103.40 ± 65.76
5	0.10–0.30	0.2549	0.3213	1.111	94.30 ± 31.21
5	0.10–0.90	0.2436	0.4065	0.626	87.32 ± 36.59
5	0.10–0.90	0.2490	0.2355	0.051	64.89 ± 15.88
5	0.30–0.90	0.3485	0.2795	0.374	121.50 ± 35.46
5	0.30–0.90	0.2408	0.4016	0.927	92.72 ± 38.39
5	0.20–0.60	0.2417	0.2148	1.411	88.18 ± 20.17
5	0.20–0.60	0.3236	0.3720	0.885	70.28 ± 28.83
10	0.20–0.60	0.2826	0.3142	1.433	77.44 ± 18.18
Mean	—	0.2572	0.3287	—	89.24 ± 30.27

Response expressed as reciprocal of $\frac{\text{urine volume at 60 minutes}}{\text{urine volume at 20 minutes}}$.

* Animals used at each dose level.
† 5% level of significance when $t = 2.179$.

P=0.05–0.1). With the intraperitoneal injection, the regression coefficient of the response on the log of the dose was 0.2684($P < 0.001$). Consequently, the intraperitoneal injection of ADS is more reliable than the subcutaneous route.

In view of the preceding, the Bliss design (1944) was utilized for the assay of ADS with the reciprocal of V60/V20 as a measure of the response to the intraperitoneal injection of Pitressin. This design permits the calculation of the relative potency of the unknown solution (potency ratio), the standard error of potency, the mean slope (b), the significance of difference between the slopes of the response-dosage curves of the standard and the unknown (t), and the index of precision (λ).

Table 3 illustrates assays performed with different numbers of animals

TABLE 4. ASSAY OF ANTIDIURETIC SUBSTANCE OF URINE

$$\frac{\text{High Dose Pitressin}}{\text{Low Dose Pitressin}} = \frac{\text{High Dose Urine Extract}}{\text{Low Dose Urine Extract}} = 3$$

(Log Dose—Response)

Subject	Sex	N*	Slope b	Index of precision λ	Significance of difference between slopes t†	Potency ratio, M ± S.E.	Urine volume, ml./24 hrs.	Pitressin equivalent	
								mU/cc.	mU/24 hrs.
D.H.	M	5	0.4523	0.1890	0.800	68.48 ± 14.02	720	0.0343	24.66
M.S.	M	5	0.3806	0.2924	0.345	99.89 ± 30.05	760	0.0295	22.39
R.M.	M	5	0.3716	0.1752	2.370	47.29 ± 10.31	780	0.0219	17.04
R.J.	M	5	0.2149	0.3235	0.100	27.32 ± 14.07	1050	0.0067	7.03
C.H.	M	5	0.2083	0.4433	0.285	40.77 ± 23.97	1280	0.0072	9.19
G.P.	F	5	0.3071	0.2524	0.326	27.89 ± 11.10	680	0.0093	6.30
J.S.	F	5	0.2205	0.4707	0.278	81.68 ± 40.28	930	0.0196	18.26
R.J.	F	5	0.3494	0.2260	0.892	87.82 ± 20.54	980	0.0202	19.76
R.G.	F	5	0.3056	0.3077	0.888	53.43 ± 19.46	1440	0.0030	12.04
B.K.	F	5	0.3194	0.4881	0.321	53.57 ± 30.96	850	0.0142	12.08
Mean	—	—	0.3130	0.3168	—	—	—	0.0166	14.88

Response expressed as reciprocal of $\frac{\text{urine volume at 60 minutes}}{\text{urine volume at 20 minutes}}$

* Animals used at each dose level.
† 5% level of significance when t = 2.179.

and varying dosage levels of Pitressin. It is apparent that the slopes of the standard and "unknown" solutions are not significantly different. Although the standard error of the potency ratio varied markedly, the precision of the assays are the same order as that of the more precise biological assays (Bliss & Cattell, 1943).

A series of experiments was performed to determine the applicability of the bioassay described herein to urine and blood plasma. In accord with others (Walker, 1939; Ham and Landis, 1942) it was observed that the removal of salts from urine by dialysis results in a 35 to 40 per cent loss of ADS. Consequently, urine was first extracted by the method of Grollman and Woods (1949) and then assayed for ADS.

In order to determine whether the activity of the ADS in urine could

be expressed in terms of Pitressin, a comparison was made between the slopes of the log dose-response curves of Pitressin and of various dilutions of an extract prepared from pooled urine. The regression coefficient of the reciprocal of V60/V20 on the log of the dose was 0.2684 for Pitressin and 0.3072 for the urine extract. Since the difference between the two slopes was found to be insignificant ($F = 0.4$, $P > 0.2$) it is permissible to express the concentration of ADS in the urine in terms of Pitressin.

Typical results of the assay of ADS in urine are given in Table 4 which summarizes the data obtained with twenty-four hour samples of urine from ten apparently healthy subjects. Two concentrations of the standard (0.2 mU and 0.6 mU Pitressin) were run in parallel with two concentrations of the urine extracts. The slopes of the standard and the unknown were essentially the same except in one instance. The values for the ex-

TABLE 5. STABILITY OF THE ANTIDIURETIC SUBSTANCE IN PLASMA. IMMEDIATELY AFTER WITHDRAWAL, THE BLOOD WAS CENTRIFUGED FOR 15 MINUTES, THE PLASMA SEPARATED AND STORED

Condition of storage	Duration of storage			
	0	3 hrs.	6 hrs.	24 hrs.
27° C.	43.5	—	—	27.6
4° C.	51.5	53.7	—	—
4° C.	63.0	54.3	78.9	—
4° C.	43.5	—	—	32.1

Expressed as mU Pitressin per 100 ml. plasma.

TABLE 6. ASSAY OF ANTIDIURETIC SUBSTANCE OF PLASMA

$$\frac{\text{High Dose Pitressin}}{\text{Low Dose Pitressin}} = \frac{\text{High Dose Plasma}}{\text{Low Dose Plasma}} = 3$$

(Log Dose—Response)

Subject	Diagnosis	N*	Slope b	Index of precision λ	Significance of difference between slopes t†	Potency ratio, M ± S.E. %	Pitressin equivalent mU/100 ml. plasma
M.S.	Normal	5	0.3999	0.2748	0.462	94.82 ± 26.78	56.9
C.H.	Normal	5	0.3699	0.1624	0.770	67.19 ± 11.93	40.3
R.P.	Normal	5	0.3322	0.2261	1.150	64.24 ± 16.01	38.5
R.J.	Normal	5	0.2580	0.3622	0.175	58.80 ± 24.33	35.3
A.B.	Normal	5	0.2261	0.3610	0.616	50.30 ± 22.03	30.2
D.H.	Normal	5	0.2483	0.4426	0.412	25.45 ± 18.32	15.3
Pooled	Normal	5	0.2012	0.4496	0.143	104.70 ± 48.90	63.0
Pooled	Normal	5	0.3680	0.3211	0.113	72.41 ± 24.93	43.5
S.W.	Hypertension	5	0.3882	0.3537	0.062	65.63 ± 25.57	39.4
P.H.	Hepatitis	5	0.4211	0.3116	0.332	69.44 ± 23.44	41.7
T.S.	Congestive Failure	5	0.5923	0.2220	0.456	49.51 ± 13.41	29.7
C.R.	Peptic Ulcer	5	0.4869	0.2633	1.760	38.84 ± 13.65	23.3
G.P.	Depression	5	0.4073	0.3076	0.263	45.53 ± 17.72	27.3
A.F.	Depression	5	0.2454	0.5685	0.013	18.23 ± 19.66	10.9
G.H.	Hysteria	5	0.3796	0.2700	0.666	36.16 ± 13.68	21.7
Mean	—	—	0.3550	0.3264	—	—	34.5

Response expressed as reciprocal of $\frac{\text{urine volume at 60 minutes}}{\text{urine volume at 20 minutes}}$.

* Animals used at each dose level.
† 5% level of significance when t = 2.179.

TABLE 7. METHODS OF ANTIDIURETIC SUBSTANCE ASSAY

Animal	Route	Method	Expression of Response	Sensitivity	Precision	Reference
Mouse	Subcutaneous	1 ml. tap water intraperitoneally followed by injection ADS. Urine measured at intervals.	Inhibition of diuresis for 3 hours.	—	—	Gibbs (1930)
Rat	Subcutaneous	16 rats divided into 4 groups. Gavaged with 5 ml. water per 100 grams followed with injections of ADS. Urine measured every 15 minutes.	Time to maximum rate of excretion	4.0 mU	Average variation 12%	Burn (1931)
Rat	Subcutaneous	90 ml. gavage with water followed by injection of ADS. Urine collected every 30 minutes.	Sodium chloride excretion	0.15 (Pituitrin)	—	Anselmino, Hoffman & Kennedy (1942)
Dog	Intravenous	Standard gavage of 250 ml. water; urine collected by catheter and recorded at 10 minute intervals. When urine flow returned to resting rate another gavage of 250 ml. of water and 1 hour later at peak of diuresis ADS injected.	Degree of inhibition of excretion	0.50 mU	—	Theobald (1934)
Rat	Subcutaneous	Burn procedure modified by priming gavage of water 3 hours before assay.	Time to maximum rate of excretion.	5.0 mU	—	Gilman and Goodman (1937)
Rabbit	Intravenous, Intramuscular, Intraperitoneal	Gavage with 1 ml. paraldehyde per kg. and 50 ml. water. Urine collected by catheter and recorded every 10 minutes. Diuresis maintained with water and paraldehyde as necessary. ADS injected during diuresis.	Rate of excretion.	Usually 0.20 mU always 0.50 mU	—	Walker (1939)
Rat	Intraperitoneal	10 sets of two rats. Gavage with 2.5 ml. water per 100 grams, followed in 2½ hours by 5 ml. water per 100 grams and injection of ADS. Urine measured every 15 minutes.	Time required to excrete 2.5% body weight.	—	—	Robinson & Farr (1940)
Dog (Diabetes Insipidus)	Intravenous	Gavage of 5 grams creatinine in 100 cc. water 2 hours before assay. Urine collected by catheter every ten minutes. Blood samples collected at hourly intervals.	Area of curve obtained by plotting creatinine U/P on time.	0.40 mU	No log dose-response	Hickey, Hare and Hare (1941)
Rat	Intraperitoneal	2.5 ml. 0.2% NaCl per 100 grams, followed 2 hours later with 5 ml. per 100 grams, and simultaneous injection of ADS. Urine volume recorded at 15 minute intervals for 3 hours.	Area of curve obtained by plotting urine excretion (ml. per 100 grams) on time. Chloride excretion.	Area—0.50 mU Chloride—0.10 mU	—	Ham & Landis (1942) and Ham (1943)
Rat	Intravenous	Initial gavage of 5 ml. alcohol-water solution per 100 grams, followed 30 minutes later by gavage with 3 ml. warm tap water per 100 grams. Needle in femoral vein and cystotomy tube tied into bladder. ADS injected when steady excretion rate obtained. Volume measured every 4 minutes during antidiuresis.	Urine volume 20 minutes after injection subtracted from that for same period prior to injection.	0.02 mU	$\lambda 0.249 \pm 0.040$*	Jeffers, Livezey, & Austin (1942)

TABLE 7—(*continued*)

Animal	Route	Method	Expression of Response	Sensitivity	Precision	Reference
Dog (Diabetes Insipidus)	Intravenous	3.0 grams creatinine in 100 cc. water administered by gavage 2 hours before beginning of assay. Retention catheter. Two clearance periods run each hour. Control period of 20 minutes and assay period 40 minutes. Urine and blood collected at appropriate intervals. Unknown assayed by being preceded and followed by some standard dose with three hours and twenty minutes shortest possible time for assay.	Concentration creatinine in urine or creatinine U/P.	0.10 mU	0.10 mU detected but estimated at 0.05 mU to 0.20 mU; an error of 100%	Hare, Melville, Chambers & Hare (1945)
Rat	Intraperitoneal	Burn's procedure. Chloride determined on urine samples representing 25% ingested water.	Time to excretion of 50% ingested water. Microequiv. chloride/min.	50% excretion time—2.0 mU Chloride—10.0 mU	—	Ralli *et al.* (1950)
Rat	Intraperitoneal	Three gavages of 3 ml. water per 100 sq. cm. of body surface, at hourly intervals. ADS injected at same time as administration of third dose of water. Urine measured one hour after second dose and every 30 minutes after third dose.	Percentage water excreted.	—	—	Birnie *et al.* (1950)
Dog (Normal & Diabetes Insipidus)	Intravenous	Water administered repeatedly by gavage so that urine volume before and after injection ADS is 5 ml. per minute. Urine collected every 5 minutes by catheter. ADS injected into vein forelimb.	ml. urine per minute.	0.25 mU	0.25 mU distinguished from 0.50 mU or 0.50 mU from 1.0 mU. Not always possible to distinguish 0.75 mU in comparison with 0.5 or 1.0 mU.	Ames, Moore, & van Dyke (1950)
Rat (Hypophysectomized)	Intraperitoneal	Gavage of 8 ml. of water per 100 sq. cm. of surface area. $\frac{2}{3}$ of water initially and $\frac{1}{3}$ at hourly intervals. Saline injected at start of third hour and ADS at start of fourth hour. Total solute concentration of urine of third and fourth hour determined.	Increase in total solute concentration of urine.	No attempt to quantify.	—	Leaf & Mamby (1952)
Rat	Intraperitoneal	This report.	Reciprocal of $\frac{\text{urine volume at 60 minutes}}{\text{urine volume at 20 minutes}}$	0.10 mU	$\lambda 0.3287$	This report

* Calculated by Bliss and Cattell (1943).

cretion of ADS ranged from the equivalent of 7.03 mU to 24.66 mU Pitressin per day. As noted in Table 4, the index of precision for the assay of urinary ADS is similar to that obtained with Pitressin.

The ADS content of blood plasma was found to be relatively low. Consequently, in order to ensure a measurable quantity of ADS in the plasma, the subjects were not permitted to ingest food or water for 10 hours prior to the sampling. Since it has been claimed that the concentration of ADS in the plasma diminished rapidly *in vitro*, the effect of storage for various periods of time and at various intervals was determined. It is apparent from the data in Table 5 that there is no significant decrease in the concentration of ADS in the plasma for at least 3 hours when the plasma is stored at the usual refrigerator temperature.

Table 6 summarizes the results of the assay of the ADS of the blood plasma of adult men treated as described above. The plasma from apparently healthy subjects contained the equivalent of from 15.3 to 56 mU Pitressin per 100 ml. of plasma. Similar data were obtained with subjects suffering from a variety of clinical disorders. As with the urine, the index of precision for the assay of plasma ADS is within the range obtained with Pitressin.

DISCUSSION

The methods that have been employed in the past for the assay of ADS are summarized in Table 7. It is evident that most of them are not subject to the application of statistical evaluation, that many are relatively insensitive, that some of the more acceptable procedures require extensive preparation or are relatively costly.

The procedure described herein obviates many of the criticisms that can be made about the methods summarized in Table 7. In accord with the more complex procedures, the proposed method utilizes each animal as its own control in that the volume of urine excreted 20 minutes after the second gavage is an index of the rate of diuresis induced by the gavage. The procedure is economical in that relatively small numbers of adult albino rats are utilized for each assay and that the animals can be used repeatedly. It is simple in that no special techniques are required other than the administration of fluids by gavage. It is sensitive in that as little as 0.1 mU of Pitressin can be detected. It is relatively precise in that not only is the coefficient of variation of the response to different doses of Pitressin relatively low but the regression coefficient of the response on the log of the dosage is quite constant while the index of precision is within the range of those of the better bioassay procedures.

The procedure described herein lends itself with ease to the assay of the ADS content of blood plasma and of urine. Pitressin can be utilized as a suitable standard since the slopes of the dose-response curves of standard

and unknown are not significantly different (Tables 4 and 6).

The number of subjects that have been studied are still too few to permit conclusions as to the normal concentration of ADS in the plasma or urine. It is evident, however, that the ADS of blood plasma is relatively stable *in vitro* and under appropriate conditions is present in measurable quantities.

SUMMARY

A relatively precise, convenient, economical procedure for the assay of Pitressin and blood and urinary ADS has been described. Rats given two gavages of 0.2 per cent NaCl are utilized for the assay. The antidiuretic response is expressed as the reciprocal of the ratio of the volume of urine excreted by 60 minutes to that excreted by 20 minutes after the second gavage. The response has a linear relationship to the logarithm of the dosage of ADS. By means of the Bliss design (1944) with 10 rats receiving a standard and 10 rats receiving an unknown quantity of ADS, the mean standard error of the potency ratio is ± 30.27 per cent and the index of precision (λ) is 0.33.

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THE THYROID AND REPRODUCTIVE PERFORMANCE IN THE ADULT FEMALE GUINEA PIG[1]

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THE general belief that the thyroid is related to reproductive function in the human female arose from the observations that the thyroid enlarges at puberty, at menstruation and during pregnancy (Garnier, 1921; Marine, 1935; Neumann, 1937; and others). This opinion was strengthened 1) by reports that sterility is frequently associated with a basal metabolic rate of −10 or lower (Litzenberg, 1926; Litzenberg and Carey, 1929), and 2) that pregnancy often occurs after treatment with thryoid preparations (Hertoghe, 1914; Veil, 1917; Huntington, 1929; Breckenridge, 1932; Mussey, 1938; King and Herring, 1939; Mason, 1939; Nicodemus and Ritmiller, 1945). Hypothyroidism has been incriminated as a causative factor in secondary amenorrhea and menorrhagia (Hertoghe, 1914; Garnier, 1921; Fluhmann and Murphy, 1941). These and other menstrual irregularities are often alleviated by thyroid therapy (Breckenridge, 1932; Mussey and Haines, 1934; Mason, 1939; Mussey, 1939; Foster and Foster, 1941; Lisser, 1942; Thompson, Thompson and Jeppson, 1947). Current clinical opinion with respect to the relationship between the thyroid and reproduction in the human female is expressed by Means (1948): "Thyroid should be prescribed in any case of infertility not traceable to some definite local cause. This applies whether the basal metabolic rate is substandard or standard." Davis (1951) states: "Thyroid medication should be tried in all women with ovarian failure for, at the present time, it is the most useful endocrine preparation we have clinically."

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Despite the abundant evidence in support of the belief that satisfactory reproductive performance depends on a normal functioning thyroid, there are conflicting reports. Baumgartner (1942) states: "Although the incidence of sterility is high among hypothyroid women the fact remains that they do become pregnant." Parkin and Greene (1943) cite five cases in which pregnancy occurred in myxedematous women without benefit of thyroid therapy. Hamblen (1940) states that there is no evidence for the effectiveness of thyroid substance in correcting ovarian failure and continues: " . . . , thyroid substance, . . . , has received less critical study of its gynecologic role than any of the various gonadotropins or sex sterols . . . "

Hyperthyroidism in the human female is not considered incompatible with pregnancy but pregnancy often makes the hyperthyroid condition worse, in some cases necessitating therapeutic abortion (Frazier and Ulrich, 1932; Taussig, 1936; Davis, 1944; Baumgartner, 1942). When disturbances in ovarian and menstrual function are associated with elevated metabolism they are sometimes favorably affected by thyroidectomy or antithyroid therapy (Wilson and Bourne, 1922; Bram, 1936; Astwood, 1951).

The relation between the thyroid and reproduction in laboratory and other domesticated mammals has received much attention. Many investigators have reported that thyroidectomy causes ovarian degeneration, arrested folliculogenesis and failure of ovulation (*rat*—Hammett, 1923; Salmon, 1936; Ross, 1938; Scow and Simpson, 1945; Leathem, 1951; *rabbit*—Hofmeister, 1894; Tatum, 1913; Kunde, Carlson and Proud, 1929; Chu, 1945; Fredrikson and Rydin, 1947; *guinea pig*—Williams, Phelps and Burch, 1941). As many or more report no effect on the ovary following thyroid removal or suppression with antithyroid drugs (*rat*—Bodansky and Cooke, 1937; Leonard and Leonard, 1937; Folley, 1938; Jones, Delfs and Foote, 1946; Krohn and White, 1950; *rabbit*—Bensen, 1902; Sax and Leibson, 1937; Krohn, 1951; *dog*—Dragstedt, Sundan and Phillips, 1924; Friedmann, 1933; Binswanger, 1936). Cyclic activity becomes irregular, prolonged or even abolished after thyroidectomy or administration of the antithyroid drugs (*rat*—Lee, 1925; Richter, 1933; Van Horn, 1933; Freedman, Wright and Webster, 1935; Ross, 1938; Smithcors, 1945; Krohn and White, 1950; *mouse*—Krohn, 1947; *guinea pig*—Williams, Phelps and Burch, 1941; *cow*—Brody and Frankenbach, 1942; *monkey*—Engle, 1944; Aranow, Engle and Sperry, 1946). Evans and Long (1921a) and Tobin (1942) indicate that an anestrous period after the operation is followed by the resumption of normal cycles.

The implication contained in much of the clinical literature, that the hypothyroid female is usually sterile, does not receive complete support from the results of experiments on laboratory mammals. Over-all fertility

may be reduced, but many of the females reproduced in spite of thyroidectomy and, in instances when measurements were made, a lowered metabolic rate (*rat*—Hammett, 1922; Bodansky and Cooke, 1937; Nelson and Tobin, 1937; Folley, 1938; Ross, 1938; Prenheim, 1940; Karnofsky, 1942; Krohn and White, 1950; *rabbit*—Parhon and Marza, 1924; Patterson, Hunt and Nicodemus, 1938; Krohn, 1951; *guinea pig*—Knaus, 1924; Marza and Marza, 1929). The antithyroid drugs apparently have a more serious effect, at least in the rat (Goldsmith, Gordon and Charipper, 1945; Jones, Delfs and Foote, 1946; Barker, 1949), nevertheless litters were born even after 10 months of treatment (Hughes, 1944; Krohn and White, 1950; Leathem, 1951).

Reports of the effects of experimental hyperthyroidism on the ovary are contradictory. Halpern and Hendryson (1935) and Ershoff (1945) state that there was a decrease in ovarian weight in the rat. Herring (1917) and Korenschevsky, Hall and Clapman (1943) noted that they were hypertrophied. The discrepancies may be explained by differences in dosage and length of treatment inasmuch as Matsumoto (cited by Hayashi, 1929) found that when small doses of thyroid were fed, an initial increase in ovarian weight was followed by atrophy. Hyperthyroidism leads to the development of irregular cycles (*rat*—Weichert, 1930; Richter, 1933; Halpern and Hendryson, 1935; Drill, Overman and Leathem, 1943; *mouse*—Cameron and Amies, 1926). The irregularity appears to be part of a general metabolic effect inasmuch as it occurred only when large doses were given (Evans and Long, 1921b; Hayashi, 1929). Treatment with desiccated thyroid or thyroxine during pregnancy is reported to lead to a high incidence of abortion and fetal death in the rat, rabbit and guinea pig (Hoskins, 1910; Gudernatsch, 1915; Döderlein, 1928, 1929; Kunde, Carlson and Proud, 1929; Weichert, 1930; Thérèsa, 1939). Chidester and Insko (1929) and Kraatz (1939), on the other hand, state that pregnancy was not prevented or interrupted.

Interest in the thyroid and reproduction has extended beyond the possible effects of hypo- or hyperthyroidism on the ovaries, on the character of the cycle, and on gestation. The results from a number of investigations have indicated that the sensitivity of many of the reproductive tissues to hormonal stimulation is influenced by the level of thyroid activity. As in other experimental work in this field, the results are contradictory and appraisal is difficult.

Thyroidectomy is reported to have augmented the ovarian response of the rat to various gonadotrophins and to anterior pituitary implants (Fluhmann, 1934; Leonard and Hansen, 1936; Bischoff, Clarke, and Epps, 1941; Salmon, 1941), whereas thyroid substances were inhibitory (Fluhmann, 1934; Tyndale and Levin, 1937; Tolksdorf and Jensen, 1939; Bis-

choff, Clarke and Epps, 1941; Warner and Meyer, 1949). The threshold of ovarian hormones required to induce menstruation in ovariectomized monkeys was first reported to be unaffected by thyroidectomy (Burford, Allen and Diddle, 1936), but Engle (1944) later reported that the threshold for estrogenic stimulation is higher in thyroidectomized animals. The administration of thiouracil to the rhesus monkey also had this effect (Aranow, Engle and Sperry, 1946). The responsiveness of the vagina of the rat to estrogens was higher following thyroidectomy and after 56 days of thiourea treatment (Langham and Gustavson, 1946), but the vaginae of mice made hypothyroid by feeding propylthiouracil were unaffected (Krohn, 1947). Thyroid preparations decreased the sensitivity of the vagina of the rat to estrogens (Reiss and Perény, 1928; Meyer and Wertz, 1938; Langham and Gustavson, 1946). Van Horn (1933) noted that three times the normal amount of estrogen was required to produce vaginal estrus in thyroid-fed rats, but he attributed this change to the high rate of estrogen metabolism rather than to any change in sensitivity of the vagina.

When the subject was considered as a whole the need for clarification was obvious. It seemed however that any new investigation should be attempted by procedures that are somewhat different from many that have been used previously. They should involve the effects of hypo- and hyperthyroidism on all aspects of reproduction from mating behavior through gestation, and both sexes should be studied simultaneously. Most important would be the relating of the reproductive performance of individuals to the level of thyroid activity as estimated from the rate of oxygen consumption and heart rate or some other measure of thyroid activity. Provided extremes of hypo- and hyperthyroidism were found to be incompatible with reproduction, such a study would lead not only to the identification of the part or parts of the reproductive process that are most closely associated with thyroid function, but also to the establishment of the range of thyroid activity within which reproduction is normal. Data for the male guinea pig have been summarized (Young, Rayner, Peterson and Brown, 1952; Young and Peterson, 1952). Those obtained for the female guinea pig are presented here.

MATERIALS AND METHODS

For any study of total reproductive performance, the guinea pig presents advantages that are not possessed by other small laboratory mammals. The character of the estrous cycle and the patterns of mating behavior are well known as they are in the rat and other species (Young, 1941; Hartman, 1945). The guinea pig is unique, however, in two important respects. Ovulation is spontaneous and, without the stimulus of coitus, is followed by the development of a fully functional corpus luteum. Except for the primates, the gestation period of 68 to 70 days is longer than that of any other laboratory mammal. About the end of the first trimester there is a transition from the ovarian to the placental

control of pregnancy (Herrick, 1928; Nelson, 1934; Ford, Webster and Young, 1951) as there is even earlier in the monkey and human female (Hartman, 1939). The result is a sequence of events from folliculogenesis through fertilization and the long gestation period which bears a close resemblance to that in the primates. Theoretically, therefore, the relationship of such a factor as the level of thyroid activity to the phases of reproduction characteristic of primates can be better tested with the guinea pig than with any other commonly used subprimate species.

The animals used in this study were healthy sexually mature females, bred in our colony and weighing at least 500 gm. at the beginning of the experiments. The temperature of the laboratory was maintained between 70° and 75° F. Food and water were available at all times. The diet consisted of a mixture of oats and rabbit pellets supplemented with alfalfa hay and fresh green vegetables daily.

In one part of the investigation reproductive capacity under conditions of hypo- and hyperthyroidism was studied. Surgical thyroidectomy was performed on a group of females at least 30 days prior to their use experimentally. The operation was carried out under numbutal anesthesia (30 mg./kg.) with the aid of a binocular dissecting microscope. An electric cautery was used in the dissection. Care was taken to avoid injuring the recurrent laryngeal nerve. No attempt was made to dissect the parathyroids free from the thyroid but quite often external parathyroids were seen at some distance from the thyroid and were left intact. As a precaution against the development of tetany, 1% calcium lactate was placed in the drinking water for 2 weeks following the operation. Fifty-nine females survived the operation and contributed data. At autopsy 45 of these were found to be free of visible thyroid rests.

The effect of propylthiouracil-induced hypothyroidism on reproductive performance was investigated in 39 females. The propylthiouracil[4] was administered as a 0.1% solution in very dilute ammonia. From records of the daily water consumption, the average consumption of the drug was found to be 25 mg. per animal per day. In previous work (Brown and Young, 1952) it was found that the ammonia had no toxic effects. The drug was administered for an average of 7 months. Tests of reproductive capacity were started 60 days after the beginning of treatment by which time the thyroids were enlarged.

Hyperthyroidism was simulated in 38 animals by subcutaneous injections of thyroxine. The animals were weighed daily and the interval between doses adjusted individually so that the weight was maintained at about 85 to 90% of the initial weight, except that allowance was made for weight-gains during pregnancy. One tenth milligram d-l thyroxine,[5] dissolved in saline, injected every 3 to 4 days was a satisfactory dose level and was continued for 8 months. Experiments were started after 30 days of treatment. Thirty-seven untreated females served as controls.

Most of the animals were observed daily for behavioral signs of estrus (Young, Dempsey, Hagquist and Boling, 1937). When a female was found in heat she was placed with a normal male and observed until copulation occurred (Young and Grunt, 1951) or until she was no longer responsive to the male. Mated females were segregated and, in order that the fertility of the mating could be established and any abortions detected, the females were observed at frequent intervals during pregnancy. Experimental treat-

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[4] The 6-propylthiouracil used in this investigation was generously supplied by Lederle Laboratories, Pearl River, New York through the courtesy of Dr. Stanton M. Hardy.

[5] The thyroxine used was supplied by Organon, Inc., Orange, New Jersey through the courtesy of Dr. K. W. Thompson.

ment was continued throughout pregnancy. Thirteen thyroidectomized females and 14 controls comprising the balance of the animals were confined with males of known fertility from 2 to 4 months.

Determinations of the rate of oxygen consumption and heart rate were made on some animals in each group at the end of the experiments. The rate of oxygen consumption was measured by use of the apparatus shown in figure 1, which is a modification of that described by Williams, Phelps and Burch (1941). The unit of comparison was the cc. of oxygen consumed per 100 gm. of body weight per hour, corrected to standard condi-

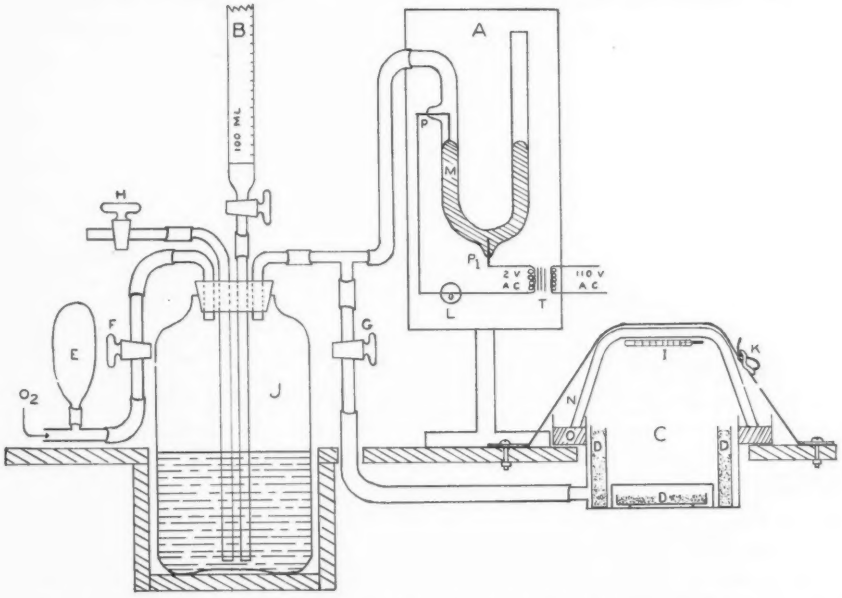

FIG. 1. Diagram of respiration apparatus. An animal is placed in the chamber (C) which contains soda lime in perforated copper baskets (D). The pyrex baking dish (N) is sealed against a sponge rubber gasket (O) by tightening web straps K. Oxygen is admitted from rubber balloon E by opening stopcocks F and G. The amount of mercury (M) in the indicating manometer (A) is so adjusted that it makes contact with electrode P when both arms of the manometer are open to atmospheric pressure. The mercury completes the circuit through the transformer (T) which lights the signal lamp (L). After a rest period of 10 minutes, time trials are taken with more than 100 ml. of oxygen in the pressure bottle (J) and with stopcocks F and H closed and G open. When the signal lamp comes on a stopwatch is started and 100 ml. of water is allowed to slowly run from the burette (B) into the pressure bottle. As pressure in the system rises the signal lamp turns off. When the lamp again comes on the stopwatch is stopped and the time required to consume a volume of oxygen equal to the volume of water added is recorded. The volume of oxygen is reduced to standard conditions from local barometric pressure and the temperature inside the animal chamber (from thermometer I). Determinations may be repeated several times without disturbing the animal. When the pressure bottle becomes full, water may be forced out by closing stopcock G and opening F and H.

tions (correction tables and sample protocol will be supplied on request). The average of three consistent determinations was taken as the rate of oxygen consumption for each animal. Heart rates were calculated from electrocardiograms taken after the animals were anesthetized with nembutal (30 mg./kg.). Loops of stainless steel wire fastened snugly around the distal portion of each limb served as electrodes. The records were taken with a Sanborn Viso-Cardiette.

The possibility of an effect of propylthiouracil-induced hypothyroidism on mating behavior was tested. The experiment also yielded data bearing on the effect of hypothyroidism on the sensitivity of the tissues mediating mating behavior to α-estradiol benzoate which, when followed by progesterone, brings guinea pigs into heat (Dempsey, Hertz and Young, 1936). Eighteen animals were ovariectomized at a median age of 76 days (range 61 to 117 days). A series of control tests for the response of the animals to the estrogen and progesterone was started on day 64 to 89 after ovariectomy. Additional tests were made on days 14, 28 and 42 of the control period. Following the last control tests, propylthiouracil was administered, as described above, to 12 of the 18 animals throughout an experimental period of 158 days. No tests were made until day 66 of the experimental period when tests of the 18 animals were resumed. Thereafter they were made on days 80, 101, 129, 146 and 158. The quantity of injected α-estradiol benzoate[6] varied from 50 I.U. to 400 I.U. per test (Table 5). The amount of progesterone was kept constant, 0.2 I.U. being given 48 hours after the estrogen. Beginning immediately after the injection of progesterone attempts to elicit the copulatory response (Young, Dempsey, Hagquist and Boling, 1937) were made at hourly intervals. Four days after the last test the animals were killed and their thyroids removed and weighed on a torsion balance. The ratio of the average relative weights for the 12 experimental and the 6 control animals was 3.75 to 1.

RESULTS

Alterations in the measures of thyroid activity that resulted from the experimental treatments are summarized in Table 1. Although the oxygen consumption rate was depressed only 6.4% below the control value by thyroidectomy and propylthiouracil treatment, it was considered that a

TABLE 1. MEASURES OF THYROID ACTIVITY IN HYPO- AND HYPERTHYROID FEMALE GUINEA PIGS

Treatment	Number of females	O_2 consumption in cc./100 gm./hr.	Heart rate in beats/min.
Controls	37	53.0 ± 1.0	271 ± 5.2
Thyroidectomy	22	49.6 ± 2.4	228 ± 9.7†
Propylthiouracil	6	49.6 ± 1.7	231 ± 13.9†
Thyroxine	10	93.8 ± 2.0†	319 ± 6.8†

The ± values are the standard errors of the means.
† The difference from the control value is significant at 1% ("t" test).

hypothyroid condition existed inasmuch as the heart rate was decreased about 16% by both treatments. The degree to which the measures of thy-

[6] Alpha-estradiol benzoate and crystalline progesterone supplied through the courtesy of the Schering Corporation, Bloomfield, New Jersey.

roid activity were elevated by thyroxine treatment clearly indicates that a condition of severe hyperthyroidism was achieved.

Cyclic activity as indicated by the frequency of vaginal openings was not significantly affected by the treatments, but it was lowest in the thyroidectomized animals (Table 2). The per cent of animals found in heat was reduced by thyroidectomy. Of the control and hypothyroid females found in

TABLE 2. EFFECT OF HYPO- AND HYPERTHYROIDISM ON CYCLIC ACTIVITY AND FERTILITY OF THE FEMALE GUINEA PIG

Treatment	Number of females	Number and % showing		Number of matings	Per cent fertile matings
		Vaginal openings	Heat responses		
Controls	39	37 94.9%	33 84.6%	30	100%
Thyroidectomy	45	36 80.0%	26* 57.8%	23	69.6%†
Propylthiouracil	39	37 94.9%	35 89.7%	31	100%
Thyroxine	38	38 100%	36 94.7%	29	100%

* The difference from the control value is significant at 2% (X^2 test).
† The difference from the control value is significant at 1% (X^2 test).

heat, approximately 90% mated. The animals given thyroxine tended to be excitable and sometimes became refractory to the male early in the test. Possibly because of this, only 80.6% of the thyroxine-treated females found in heat mated. There were no sterile matings except among the thyroidectomized females.

The per cent of pregnancies terminating the sixty-sixth day or later was not influenced by hypo- or hyperthyroidism (Table 3). The length of gestation and litter size were likewise unaffected. The per cent of offspring born

TABLE 3. EFFECT OF HYPO- AND HYPERTHYROIDISM IN FEMALE GUINEA PIGS ON THE COURSE OF GESTATION AND THE NUMBER AND CONDITION OF THE OFFSPRING

Treatment	Number and % pregnancies terminating 66th day or later	Average length of gestation in days	Average litter size	Number of young	Per cent born alive
Controls	26 92.8%	68.8	3.2	110	73.6%
Thyroidectomy	8 88.9%	66.8	3.1	47	48.9%†
Propylthiouracil	27 90.0%	69.4	2.7	92	67.4%
Thyroxine	24 82.8%	67.4	3.4	100	98.0%†

† The difference from the control value is significant at 1% (X^2 test).

alive was markedly reduced by thyroidectomy of the mother thus indicating further impairment of the ability of such animals to reproduce. On the other hand, the per cent of young born alive to the thyroxine-treated mothers was significantly higher than in the controls. This increase is of

interest in connection with a study reported by Chu (1945). He found that the administration of desiccated thyroid to thyroidectomized rabbits in which superovulation had been induced by chorionic gonadotrophin was effective in retaining the supernormal number of fetuses in a normal condition until term. Without the thyroid treatment the number of fetuses at term was normal but a high per cent were born dead.

Propylthiouracil, although suppressing oxygen consumption and heart rate to points comparable with these measures of thyroid activity in surgically thyroidectomized animals, was without effect on reproduction except for the condition of the young. The newborn had extremely enlarged thyroids (Fig. 2) similar to those described by Albrieux, Estefan and

FIG. 2. A newborn male guinea pig showing the enlarged thyroid gland characteristic of animals born to mothers treated with propylthiouracil during pregnancy.

Gonzalez (1946). The development of these goiters is attributed to the placental passage of propylthiouracil (Peterson and Young, 1952). Although born alive, many of these goitrous newborn lived but a short time. Had the colony not been under close observation at frequent intervals, many of these would have been recorded as stillbirths. Sufficient animals survived the neonatal period to enable us to follow their growth and maturation. The thyroids which at birth were 84 times heavier than the thyroids of controls underwent a rapid involution, but even after 8½ months were almost 5 times heavier than those from controls. The goiters had no apparent effect on growth and sexual maturation. Females goitrous at birth showed cyclic estrous activity as soon as normal females and gave birth to normal offspring. The development of sexual maturity in males goitrous at birth was not different from that of normal males (Webster and Young, 1951).

In the analysis of the data as a whole it seemed desirable to compare the level of thyroid activity in females that reproduced with the level in fe-

males which did not reproduce (Table 4). When oxygen consumption and heart rate of animals that failed to reproduce are compared with these measures of thyroid activity in animals that reproduced, it is seen that they were often very similar. Clearly, therefore, individuals react differently to given levels of thyroid hormone. The data also reveal something of the range of thyroid activity within which reproduction is possible.

TABLE 4. MEASURES OF THYROID ACTIVITY IN FEMALE GUINEA PIGS THAT REPRODUCED AND IN THOSE THAT FAILED TO REPRODUCE

Treatment	Reproduced			Failed to reproduce		
	No.	O_2 consumption cc./100 gm./hr.	Heart rate beats/min.	No.	O_2 consumption cc./100 gm./hr.	Heart rate beats/min.
Controls	28	52.9±1.2	272± 6.6	9	53.3±2.0	268±6.4
Thyroidectomy	9	50.0±2.5	258±12.0	13	49.3±2.4	202±6.9†
Propylthiouracil	4	51.0±2.3	238±25.1	2	46.7±0.7	222±5.2
Thyroxine	6	93.5±3.0	316± 8.3	4	94.1±2.7	330±1.2

The ± values are the standard errors of the means.
† The difference from the corresponding value for animals that reproduced is significant at 1% ("t" test).

In the 14 thyroidectomized females in which thyroid rests were found at autopsy, oxygen consumption and heart rate were intermediate between the controls and the thyroidectomized animals that were free of thyroid tissue. They retained this intermediate position with respect to the per cent of cyclic vaginal openings, the per cent found in heat, and the per cent of fertile matings. In other respects they were more nearly like the controls.

The data bearing on the effects of propylthiouracil-induced hypothyroidism on the responsiveness of the tissues mediating mating behavior to estrogen are summarized in Table 5. The shorter heat periods during the experimental period when 50 I.U. α-estradiol benzoate were given suggested that the sensitivity of the tissues mediating mating behavior had decreased.

TABLE 5. SENSITIVITY TO ESTROGEN OF PROPYLTHIOURACIL-INDUCED HYPOTHYROID OVARIECTOMIZED GUINEA PIGS AS MEASURED BY THE LENGTH OF HEAT IN HOURS

I.U. Estrogen		Day of control period				Day of experimental period					
		1	14	28	42	66	80	101	129	146	158
50	Exp.	5.9±0.7	4.6±1.0		4.2±1.0	3.7±0.8		3.3±0.6	1.9±0.6		
	Con.	4.3±1.4	5.2±1.7		4.0±0.9	3.2±1.0		2.7±1.2	1.5±1.0		
100	Exp.			5.8±0.9			5.8±0.7			3.0±0.8	
	Con.			7.3±0.9			7.8±1.1			3.7±1.1	
400	Exp.										6.3±0.9
	Con.										7.5±1.2

The ± values are the standard errors of the means.

Confirmatory evidence seemed to be provided by the results obtained when larger quantities of estrogen were injected. Each time 100 I.U. α-estradiol benzoate were given the induced heat period was longer than it was during the preceding test when 50 I.U. were given, and in the last test when 400 I.U. were given, the average length of heat was greater than it was 12 days previously when 100 I.U. were given.

The fact that the decrease shown during the 158 days of the experimental period was not greater in the 12 animals given propylthiouracil than it was in the 6 untreated females precludes the possibility that the change is attributable to hypothyroidism. The results obtained from this more carefully controlled experiment will correct the impression conveyed in an earlier abstract (Innes, Young, and Webster, 1947) that the decreased length of heat was attributable to propylthiouracil treatment. It is now thought that the change is perhaps a part of the aging process, but the possibility should be tested by supplementary experiments.

DISCUSSION

The data presented above are believed to have a significance for the problem of the relationship between the thyroid and reproductive performance in the female guinea pig that can be summarized briefly. The degree of hyperthyroidism that was attained, which was close to the limits of tolerance for many animals, seems to have had no deleterious action on any phase of reproduction from folliculogenesis through mating and the course of gestation. On the other hand, the per cent of young born alive to females given thyroxine was much higher than in any other group.

Impairment of reproduction was seen in many but not all hypothyroid females. The nature of the effect depended on the manner in which hypothyroidism was produced. In the surgically thyroidectomized animals the frequency of cyclic vaginal opening was low, the per cent of animals found in heat was reduced, the per cent of sterile matings was increased, and the per cent of young born dead was high. The only detectable effect of propylthiouracil-induced hypothyroidism, which depressed thyroid activity to the same level as surgical thyroidectomy, can be attributed to the placental passage of the drug. Extremely large goiters in the newborn may have accounted for the abnormally high per cent of neonatal deaths.

Comparison of these results with those obtained during work with the male (Young, Rayner, Peterson and Brown, 1952; Young and Peterson, 1952) is of interest. In neither sex did the degree of hyperthyroidism produced have any effect on over-all reproductive performance, but in both sexes fertility was decreased following surgical thyroidectomy. The decrease was greater in the female than in the male, although thyroid activity was depressed more in the male. This would seem to indicate that repro-

ductive processes are more dependent on normal thyroid function in the female.

The results obtained when the male was being studied indicated that the range of thyroid activity within which reproduction can occur is wide, and that individuals react differently to low levels of thyroid hormone. Within the limit that the level of thyroid activity has been varied in the female the same conclusions are suggested, but we cannot be certain of the reaction of the female to the extreme hypothyroidism produced in the male (Young and Peterson, 1952) until comparable experiments have been performed.

A point for point comparison of the results obtained during this study with those reported by other investigators would be difficult, but certain generalization is suggested. The conclusion that in the guinea pig chronic hyperthyroidism is without effect whereas hypothyroidism impairs reproductive performance is consistent with much of the experimental and clinical literature we have cited. We are impressed, however, by the reports that hypothyroid females of other species reproduce (Folley, 1938; Krohn and White, 1950; Krohn, 1951; Leathem, 1951). These reports and the numerous older contradictory claims cannot all be ascribed to inadequate controls and species differences. Were attempts made in a comparative study of a number of species to define the limits of thyroid activity beyond which reproduction is impaired or fails, and to determine the nature of the impairment as these limits are approached, it is believed that clarification of the problem might be achieved.

SUMMARY AND CONCLUSIONS

The reproductive performance of adult female guinea pigs under conditions of hypo- and hyperthyroidism was studied. The degree of alteration in the level of thyroid activity was estimated from oxygen consumption and heart rate determinations.

Surgical thyroidectomy which decreased the rate of oxygen consumption 6.4% and heart rate 18% decreased the frequency of cyclic vaginal openings, the per cent of animals found in heat, the per cent of fertile matings, and the per cent of young born alive.

Propylthiouracil-induced hypothyroidism, which reduced the measures of thyroid activity to a degree comparable with that following thyroidectomy, was without detectable effect on reproductive performance except that large goiters were present in the young born to such females. In ovariectomized animals propylthiouracil-induced hypothyroidism did not alter the responsiveness of the tissues mediating mating behavior to α-estradiol benzoate.

The reproductive performance of females made hyperthyroid by doses of thyroxine close to the limits of tolerance was if anything better than in the

controls, particularly with respect to the per cent of young born alive.

There is a wide range of thyroid activity compatible with reproduction, and individuals react differently to given levels of thyroid activity.

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THE INHIBITORY ACTION OF VARIOUS STEROIDS ON THE HOOKER-FORBES ASSAY FOR PROGESTERONE[1]

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THE effects that one hormone may have upon the physiological action of another is of considerable interest and importance. Both "synergistic" and "antagonistic" interactions have been observed, and some of these have been discussed recently at some length by Courrier (1951). The possibility that the effect produced by one steroid hormone may be augmented or inhibited by another is of particular significance in procedures for quantitative assays.

Hooker and Forbes (1947, 1949) described a microbioassay for progesterone which they considered specific. They found that the accuracy of the assay was unaffected by the presence of estrogen (Hooker and Forbes, 1947). Inasmuch as the response observed by Hooker and Forbes involved changes in the connective tissue of the endometrium of the mouse uterus, this independent action of progesterone seemed to be in conflict with the observations by Morel (1948) on the antagonistic action of estrogen on the decidual reaction in castrated mice. Morel found that estradiol inhibited the action of progesterone in supporting deciduomata whenever the ratio of injected estrogen to progesterone was 1:200 or greater. Apparently this connective tissue response to progesterone is not exempt from the inhibitory action of estrogen as has been observed in a number of reactions in several species of laboratory mammals. The marked sensitivity of the connective tissue cells of the endometrium in mice to progesterone and its usefulness as an assay method indicate the desirability of further study of the influence of estrogen on the reaction of the stromal nuclei.

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MATERIALS AND METHODS

The procedure was essentially that described by Hooker and Forbes (1947), and all slides prepared were examined while coded, thus eliminating any element of subjectivity. Occasionally, at autopsy, the injected segment of the uterus was found to be swollen, and especially so when estrogen was administered. Such distension rendered the uteri unusable for the purpose of the experiment as the shape of the stromal nuclei was distorted. In such instances no data were collected, the response being indicated as "DIS."

Two strains of mice were used. One was the CHI strain also used by the Yale group, and the other the Rockland Farms' Swiss albino strain. No apparent differences in the responses of the strains were observed. In the initial experiments the hormones were dissolved in a few drops of absolute alcohol and diluted to the desired concentrations by the addition of Ringer's solution. Later the steroids were injected in sesame oil. No differences were observed in the results when either vehicle was used. Routinely the various solutions were incubated overnight at 37° C. to assure complete solution and mixing of the hormones.

TABLE 1. INHIBITION OF THE HOOKER-FORBES ASSAY FOR PROGESTERONE BY ESTROGEN: ROCKLAND FARMS WHITE MICE

Solvent used	Progesterone, μg./ml.	Estradiol 17-beta, μg./ml.	Ratio estradiol: progesterone	Hooker-Forbes assay response*
Distilled Water	Saturated	0.0	—	+
	Saturated	0.1	?	−
Mammalian Ringer's	2	0.0	—	+, +
	2	0.01	1:200	−, DIS
	2	0.1	1:20	−, DIS
	2	1.0	1:2	−, −
	2	10.0	5:2	−, −
Sesame Oil	5	0.0	—	+, +, DIS
	5	0.025	1:200	+, −S, −
	5	0.25	1:20	−, −, −

* + Positive Hooker-Forbes response in rhe stromal nuclei.
−S The stromal cells are enlarged and stimulated, but other criteria for a positive response are not met.
− Negative Hooker-Forbes response.
DIS No data obtainable due to distension of injected segment of uterus.

EXPERIMENTAL RESULTS

The data obtained in the preliminary experiments in which Swiss albino mice were used (Table 1) were duplicated and extended on a quantitative basis in CHI strain mice (Table 2). Solutions of estradiol 17-beta and progesterone in ratios ranging from 1:5000 to 1:2 were assayed. The inhibitory action obtained in each series of assays was determined by comparing the reaction given by the same amount of progesterone as that used in the mixture when injected alone. When the concentration of estrogen was low as compared with progesterone in the mixed solutions, positive nuclei were observed in the stromal connective tissue. These positive reactions were

given by mixtures having estradiol: progesterone ratios of 1:5000 to 1:300. When the ratio of estradiol to progesterone was 1:200, or greater, the modification of the atrophic stromal nuclei into a positive response was inhibited (Table 2).

Some variation in the reaction of the stromal nuclei to the intra-uterine injection of progesterone had been reported by Hooker and Forbes (1947), and we, too, encountered a degree of variability. A "false negative" was obtained with an intra-uterine injection of 5μg. progesterone per ml. sesame oil, and a "false positive" with a test mixture containing estradiol and progesterone in a ratio of 1:20 (Table 2). Since many animals were used, the occasionally anomalous responses of the stromal nuclei are not significant.

TABLE 2. INHIBITORY EFFECT OF ESTROGEN ON THE HOOKER-FORBES ASSAY FOR PROGESTERONE IN SESAME OIL SOLUTIONS: CHI STRAIN MICE

Progesterone μg./ml.	Estradiol 17-beta, μg./ml.	Ratio estrogen: progesterone	Hooker-Forbes assay response
2	0.0	—	+, DIS
2	0.01	1:200	−S, −
2	0.1	1:20	−, −
2	1.0	1:2	−, −
5	0.0	—	+, +, +, +, +, +, −
5	0.001	1:5000	+, DIS
5	0.01	1:500	+, +
5	0.013	1:400	+, +
5	0.017	1:300	+, +
5*	0.025	1:200	−S, −, −, −, −, −
5	0.25	1:20	+, −S, −, −, −, −
5*	0.025	1:200	−S, −, −, −, −, −
10	0.05	1:200	−, DIS
20	0.1	1:200	−S, −

* Duplicated for comparison.

Apparently the ratio of 1:200, or thereabouts, is the critical balance of estradiol and progesterone for this reaction. The ratio is effective over a range of absolute amounts of progesterone since solutions of 2.0, 5.0, 10.0 and 20.0 μg. per ml. containing the appropriate amount of estradiol to bring the ratio of estradiol to progesterone to 1:200 uniformly failed to elicit positive responses (Table 2).

Since estradiol had proven antagonistic to progesterone in the intra-uterine assays, we investigated the effects of some other steroids. The widely used synthetic estrogen, diethylstilbestrol, markedly inhibited the Hooker-Forbes assay when added to the test sample. Testosterone, when present in a concentration of 10.0 or 20.0 μg. per ml. of a solution containing 5 μg. progesterone, also seemed to prevent the transformation of the stromal cells. Intra-uterine injections of a solution of 5 μg. progesterone and 10 μg. desoxycorticosterone acetate (DCA) per ml. sesame oil gave a

very variable response. The inhibition caused by DCA was not as evident as that resulting from estradiol, for positive as well as negative assay responses were obtained (Table 3). It was noted that intra-uterine injections of DCA tend to distend the uterine segment.

TABLE 3. EFFECT OF SEVERAL STEROIDS ON THE HOOKER-FORBES ASSAY FOR PROGESTERONE: CHI STRAIN MICE

Progesterone, μg./ml.	Solvent	Steroid	Amount, μg./ml.	Hooker-Forbes assay response
5	Sesame oil	Stilbestrol	2.5	−, −, DIS, DIS
5	Sesame oil	Testosterone	10.0	−S, −, −
5	Sesame oil	Testosterone	20.0	−, −
5	Sesame oil	Desoxycorticosterone acetate	10.0	+, −, −, DIS
5	Distilled water	Desoxycorticosterone glucoside	10.0	+, −

DISCUSSION

The primary implication of these results is concerned with the specificity of the intra-uterine micro-assay for progestational activity. These data indicate that the assay method is not accurate when the ratio of estradiol to progesterone attains a critical level. It was found that testosterone and desoxycorticosterone also seem to obscure the assay response; but whether they are ever present in sufficiently high concentrations in the blood under physiological conditions to affect the bioassay is still open to investigation.

Perhaps it should not be surprising that the effective inhibitory ratio of estrogen to progesterone is 1:200. Hooker (1945) has stated that the cytological change of the stromal nuclei, on which the assay is based, occurs normally in the formation of a deciduoma in the mouse, and Morel (1948) has found this ratio of 1:200 to be effective in preventing the production of the decidual reaction. Although Morel employed subcutaneous injections, it is significant that the results were of a similar nature.

Two characteristics of the inhibition of the reaction of the stromal nuclei seem relevant to the quantitative nature of the bioassay: estradiol blocks the action of progesterone when present in a ratio equal or greater than 1:200; and, this inhibitory ratio seems independent of the absolute amounts of the two hormones. Then, since the inhibition by estrogen occurs in an "all or none" fashion, the assay of a solution containing a critical ratio of estrogen and progesterone would result in a "false negative" rather than an apparently lower concentration of progesterone. Consequently, a positive assay reaction resulting from the serial dilution technique would closely reflect the actual concentration of progesterone present in a mixed solution.

The most important consideration of these results is whether the inhibitory action of estrogen would affect assays for progesterone under

normal physiological conditions. For example, assay levels of progesterone in the plasma of pregnant women range around 5 μg. per ml. (Forbes, 1951). Similarly, human pregnancy blood levels of estrogen, measured as equivalents of estradiol-17-beta, range from 0.2 to 0.5 μg. per 100 ml. whole blood (Szego and Roberts, 1946). Allowing for corrections with respect to hematocrit and the volume of blood that was extracted, the respective ratios under these conditions appear at least to be of an order of magnitude permitting for interference with the quantitative accuracy of the assay.

In a recent paper, however, Forbes (1951) has suggested the possibility that progesterone may not be the chemical entity responsible for normal progestational activity. If this be true, then estrogen may have a different relation to the "true progestational hormone" than it has to progesterone. To our knowledge, however, since no progestational hormone other than progesterone has been isolated from physiological sources, and since virtually all the progestational activity in human placental extracts can be attributed to progesterone (Salhanick, Noall, Zarrow, and Samuels, 1952) we have assumed that, whatever the nature of a supposed progestational hormone, it would react similarly to progesterone.

The Hooker-Forbes assay for progesterone seems to be by far the most sensitive and specific yet advanced. Our knowledge, however, of the exact amounts of the various estrogens in the blood of animals during normal estrus or menstrual cycles and during pregnancy is still deficient. Until such information becomes available it cannot be stated conclusively whether or not the micro-bioassay for progesterone is quantitatively valid under physiological conditions.

SUMMARY

Estradiol-17-beta inhibits the positive response elicited by progesterone in the Hooker-Forbes bioassay when present in estradiol:progesterone ratios of 1:200 or greater. Stilbestrol and testosterone also prevent the typical progestational response, and application of either the acetate or glucoside of desoxycorticosterone obscures the action of progesterone on the stromal nuclei. These observations have a direct bearing on the quantitative assay of progesterone in mixtures containing other steroid hormones.

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EFFECT OF THYROID PREPARATIONS AND IODIDE ON SALMONIDAE[1]

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UNTIL recently, the common belief was that, in fish, the thyroid hormone did not have a metabolic effect similar to the one observed in mammals, or even that it had no effect at all. Thus, Etkin and his collaborators were unable to detect any effect of thyroid extract or thyroxine administration on the oxygen consumption of the goldfish, *Carassius auratus*, and toadfish, *Opsanus tau* (Root and Etkin, 1937; Etkin *et al.*, 1940). Hasler and Meyer (1942) experimenting with the goldfish, *Carassius auratus*, also obtained negative results with thyroxine. However, an increase in the oxygen consumption of the parrotfish, *Bathystoma*, was observed after treatment with parrotfish thyroid extract by Smith and Matthews (1948), but only in the larger animals. Krokert (1936) observed changes in the shape of the fins of guppies, *Lebistes reticulatus*, fed thyroid extracts. Smith and Everett (1943) were unable to reproduce these results, while Hopper (1950, 1952) found that thyroid treatment accelerates gonopod differentiation in this species. Landgrebe (1941) injected thyroid extracts into two Atlantic salmon, *Salmo salar*, at the parr stage and produced a paling of their integument. A similar result was obtained in the parr of the rainbow trout, *Salmo gairdnerii*, by Robertson (1949) and in the parr of the Atlantic salmon, *Salmo salar*, by the present authors (La Roche *et al.*, 1949, 1950)[1] and by Hoar (1951).

A systematic study of the effect of thyroid preparations was undertaken in Atlantic salmon parr, as well as in younger (fry) and older (smolt) salmon. Brook trout, *Salvelinus fontinalis*, at the parr stage were examined for comparison. The thyroid preparations produced a considerable increase in the thickness of the skin layers as well as a decrease in the pigmentation of these various animals. Since similar changes were observed during the transformation of parr into smolt under natural conditions, it seems quite possible that the secretions of the thyroid gland play a role in this transformation, as was suggested by Hoar (1939), Landgrebe (1941), Fontaine (1948), Robertson (1949), and La Roche *et al.* (1949, 1950).

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[1] Preliminary reports of these results were presented before the American Association of Anatomists (La Roche, 1949, 1950 and 1951).

TECHNIQUES

Most of the work with thyroid preparations was done in the young Atlantic salmon, *Salmo salar*, at the parr stage. A middle sized parr may be readily identified by means of characteristic lateral bars (Fig. 7), but there may be some difficulty in recognizing the youngest and oldest animals at this stage. Here, parr were defined arbitrarily as measuring from 5–15 cm. in length.

Three series of experiments were performed. The first two series were carried out at the Fish Hatchery of the Laurentides in St. Faustin, Quebec, where the parr were kept in tanks measuring 144 by 18 by 7 inches. The third series were carried out at the Gaspé Fish Hatchery in Gaspé, Quebec, where the parr were kept in tanks measuring 126 by 15 by 4 inches. The rate of flow of water was approximately 4 liters per minute in all cases. The temperature of the water—estimated twice a day—varied from 8–10° C. in the first, 10–12° C. in the second, and 9–13° C. in the third series of experiments. Once a week, the animals of each group were weighed together.

First Series

This series, extending from June 5 to August 23, 1948, consisted of 4 simultaneous experiments on hatchery reared parr to study the effects of 1) thyroxine, 2) commercial thyroid extract, 3) dry thyroid preparation, and 4) iodide.

The first experiment of this series included two groups of 30 parr each, i.e., a control group injected with 0.05 cc. of saline twice a week intramuscularly under light urethane anesthesia, and a treated group similarly injected with 200 μg. of sodium thyroxinate in a volume of 0.05 cc. An excess of fresh ground beef liver was provided every other day as food for these animals. In both groups, some died due to growth of fungi (*Saprolegna*) at the injection sites. Nineteen control and 17 treated fish survived in good condition until the end of the experiment.

TABLE 1. EFFECTS OF VARIOUS THYROID PREPARATIONS ON SALMON PARR
(*1st series*)

	Number of animals	Average body weight (gm.)			Degree of enophthalmos	Overall pigmentation
		Initial	Terminal	Weekly increase		
Control	19	8.5	15.0	1.10	0	+±
Sodium Thyroxinate	22	7.6	11.5	0.65	+	+
Control	17	5.0	10.2	0.60	0	+++
Thyroid Extract	17	6.7	9.4	0.36	+	+
Control	30	7.6	11.5	0.65	0	+++
Beef Thyroid	30	6.9	10.6	0.61	+	+

The second experiment included a control group of 17 parr fed beef liver and another group of 17 parr fed a half and half mixture of beef liver and commercial thyroid extract (Casgrain & Charbonneau Ltd.) known to contain 0.3% iodine. All the control and 16 thyroid treated fish survived until the end of the experiment.

The third experiment consisted of a control group of 30 parr fed beef liver and another group of 30 parr fed a mixture of 25% beef liver and 75% pulverized beef thyroid (obtained fresh from Canada Packers slaughter house and dried *in vacuo* at 90° C.). The number of survivors in these two groups was 30 and 29 respectively.

The fourth experiment consisted of 3 groups of 20 parr each fed every other day on beef liver. The first group was used as control. The other two groups of animals were kept in an environment enriched with iodide by dropwise addition of a solution of sodium or potassium iodide to the water flowing into the tanks. The rate of iodide addition was such that the water of the tank provided for the second group contained 10 parts and that for the third group, 60 parts of iodide per million (p.p.m.). In this fourth experiment, 20 fish survived in the first, 20 in the second and 18 in the third group.

TABLE 2. EFFECTS OF IODIDE ON SALMON PARR
(*1st series*)

	Number of animals	Average body weight (gm.)			Pigmentation	Histological aspect of thyroid
		Initial	Terminal	Weekly increase		
Control	17	5.0	10.2	0.60	+++	Stimulated (Fig. 6)
Iodide (10 p/1,000,000)	17	4.7	8.7	0.44	++±	Resting (Fig. 7)
Iodide (60 p/1,000,000)	16	5.5	8.7	0.41	++	Resting

At the end of this series of experiments, typical live fish of the groups receiving thyroid preparations and appropriate controls were photographed (Fig. 3). The fish were sacrificed by being placed in a solution of urethane. Pigmentation was assessed subjectively (Tables 1 and 2). The thyroid regions were fixed in Bouin's fluid, and subsequently sectioned and stained with Masson's trichrome.

Second Series

This series, extending from July 18 to December 17, 1949, consisted of a single experiment designed to confirm the effect of thyroid extract on hatchery reared parr of various sizes.

The experiment included two groups of 30 parr each, a control group fed a mixture of horse liver and spleen, and a treated group fed dried beef thyroid mixed with about half its weight of the liver and spleen combination given the controls. Both groups were subdivided into 3 subgroups of 10 animals each, whose lengths were 5–8, 8–11, and 11–14 cm. respectively. Initially and at 10 weeks, the fish of each subgroup were weighed and their lengths were measured (Table 3). A few animals were also sacrificed at the end of this 10-week period of treatment. The experiment was terminated 5 months later. Several live animals of each subgroup were photographed, from the side and from the top (Figs. 4–10). They were then sacrificed in urethane and their lengths recorded. For histological study, the head, operculum, caudal and pectoral fins were fixed in a neutral alcohol-formalin mixture, and the internal organs in Bouin's fluid. In addition to routine stains, the von Kossa method for the detection of calcium salts was also used.

Third Series

This series, extending from June 27 to August 31, 1950, included young salmon at the fry stage (3–5 cm. in length), at the parr stage (5–15 cm. in length), and at the smolt stage (15–25 cm. in length). The goal of this experiment was to determine whether

thyroid feeding affected fry and smolt as well as parr, and also to obtain quantitative data on skin thickness and pigment cell population after treatment.

The first experiment consisted of two groups of 500 yearling salmon fry each, a control group fed beef liver, and a treated group fed a diet consisting of half beef liver and half dried beef thyroid. The second experiment consisted of similar control and treated groups of 100 parr each. In the third experiment, both groups consisted of 20 smolt. The fry were fed 5 times a week, the parr and smolt 3 times a week. (The parr were obtained by seining the York river, while the fry and smolt had been raised in the Gaspé hatchery.)

Furthermore, brook trout parr, 5–8 cm. in length were used in a fourth experiment to see whether thyroid feeding was effective in species other than salmon. Fifty trout were kept as controls and given a diet of ground beef liver, while another 50 were given beef

TABLE 3. INFLUENCE OF TREATMENT WITH THYROID EXTRACT ON BODY WEIGHT AND LENGTH OF SALMON PARR

(*2nd series*)

Sub-group number	Range of body length at beginning of experiment		Average body length (cm.)			Average body weight (gm.)		
			Initial	Terminal	Weekly increase	Initial	Terminal	Weekly increase
1	5–8 cm.	Control	7.2	8.6	0.14	3.1	6.2	0.31
		Treated	7.3	8.7	0.14	3.2	5.7	0.25
2	8–11 cm.	Control	9.3	10.5	0.12	7.0	10.9	0.39
		Treated	9.3	11.2	0.19	7.1	11.8	0.47
3	11–14 cm.	Control	12.2	13.6	0.14	15.7	25.3	0.96
		Treated	12.2	13.6	0.14	17.2	24.5	0.73

thyroid mixed as above with half its weight of beef liver. The animals were fed 3 times a week.

The experiment continued 8 weeks. Some of the animals were sacrificed and their tissues fixed in Bouin and stained with Masson's trichrome. Other animals were placed in 10% formalin, and later photographed (Figs. 17–22).

The number of *pigment cells* per unit area of dorsal and ventral skin was estimated. For that purpose, 4 treated and 4 control animals from each group were injected with a solution containing 1/1000 of adrenalin to produce a contraction of the pigment cells, and thus facilitate counting them. According to the size of the animal the dose varied from 0.05 up to 1.50 cc. of the adrenalin solution. The fish were sacrificed approximately 10 minutes after injection. Pieces of skin were taken from the dorsal and ventral regions midway between the dorsal and adipose fins, care being taken to include all layers down to the underlying muscles. The skin samples were fixed in 50% alcohol, dehydrated in successively increasing alcohol concentrations up to absolute, cleared in xylene and mounted whole in cedar oil.

Pigment cells were counted in the dorsal and ventral regions. Areas measuring 0.2 sq. mm. were projected onto paper by means of a prism adapted to the ocular of the microscope. It was known that pigment cells are located in the derma, where they form two layers, one on the inner surface of the epidermis and another on the outer surface of the subjacent bones or muscles. The cells of both layers present in the projected area

were drawn and counted. This procedure was repeated in 4 dorsal and 4 ventral areas in each animal. Since 4 animals were examined per group, there was a total of 16 determinations for both the dorsal and ventral region of each group (Table 4).

To measure the *thickness of the skin*, 5 or 6 animals were used per group. The sections were projected at a magnification of 100 times, using a 45° prism adapted to the eyepiece of the microscope. The outline of the epidermis as well as that of the subjacent derma was traced on paper. This was done in 4 different regions of the body (ventral region of the head, dorsal region of the head, body and tail fin). By cutting along the drawn lines, pieces of paper corresponding to epidermis and derma were obtained. These were weighed on an analytical balance, thus yielding the relative weights of the two layers.

Five pieces of the paper used for projection were cut so as to measure 10,000 sq. mm. and weighed. The average weight divided by 10,000 gave the weight of 1 sq. mm. of paper. This made it possible to reexpress the relative weights of the cut-outs of skin layers as surface areas of the sections of each layer.

TABLE 4. INFLUENCE OF THE ADMINISTRATION OF BEEF THYROID ON THE NUMBER OF PIGMENT CELLS PER UNIT AREA OF VENTRAL AND DORSAL SKIN IN YOUNG SALMON (FRY, PARR, SMOLT STAGES)

Stages	Treatment	Average body weights gm.			Ventral region		Dorsal region	
		Initial	Terminal	Weekly increase	No. of pigment cells	Value of P	No. of pigment cells	Value of P
Fry	Controls	0.5	0.8	0.037	6.7	$P<0.001$*	79.6	$0.4>P>0.3$
	Beef thyroid	0.5	0.8	0.037	1.2		75.7	
Parr	Controls	5.1	6.3	0.15	3.2	$P<0.001$*	89.1	$0.01>P>0.001$*
	Beef thyroid	5.1	6.9	0.23	0.9		64.3	
Smolt	Controls	40.3	77.5	5.3	6.7	$P<0.001$*	91.7	$0.1>P>0.05$
	Beef thyroid	38.6	62.1	3.3	1.2		82.2	

The total length of the basement membrane of the segment of epidermis under investigation was then measured in each area by following the outline of its projection with a map reader and dividing by the magnification. It was assumed that the surface area of the projected epidermis was a rectangle, the base of which was formed by the basement membrane. By dividing the area of the rectangle by the length of the basement membrane it was possible to obtain its height, which represents in fact the mean thickness of the epidermis. The mean thickness of the derma was similarly determined on the assumption that its projection was a rectangle of length equal to the basement membrane. The values for the thickness of the epidermis, expressed in μ, are represented as block diagrams in Fig. 23. The corresponding values for the derma are similarly shown in Fig. 24. In addition, the thickness of the whole skin was obtained by adding the values for epidermis and derma, and was then plotted against the length of the animals (Fig. 25).

Finally, there seemed to be an enlargement of the space located between the eyeballs, at least in trout. It was, therefore, decided to measure interorbital distances on cross sections of the head at the level of the eyeballs. The interorbital distances were measured with the help of an ocular micrometer.

RESULTS

First Series

In the first, second and third experiments of this series, the effects of various thyroid preparations on salmon parr was examined; while in the fourth experiment, the effects of iodide were investigated.

Thyroid treatment. The *growth* of the treated animals was somewhat less rapid than that of the controls, as shown by the mean weekly increase in body weight (Table 1).

The *pigmentation* in the treated fish was less than in controls. The difference became distinguishable 3 weeks after the beginning of treatment. The pallor was moderate in thyroxine-treated fish and pronounced in those fed commercial thyroid extract or beef thyroid (Fig. 3; Table 1). There was a fading of the lateral bars. Some pallor persisted in 6 animals kept for 3 months after cessation of the thyroid treatment.

To examine whether the pallor was due to a contraction of the pigment cells, the animals were anesthetized with urethane—a procedure which gives a moderate expansion of these cells. Some darkening resulted in both control and treated animals. However, the difference between control and treated remained as pronounced as before urethane anesthesia and, therefore, appeared independent of the degree of pigment cell expansion.

Other changes observed at the end of the period of thyroid treatment consisted of protrusions on top of the head, apparently at the level of the dermethmoid and supraoccipital bones. When viewed from above, the animals showed a lateral protrusion of the opercula (Fig. 3). The eyes of the treated animals appeared more deeply embedded in the tissues of the head than those of the controls (Fig. 3)—a condition referred to as enopthalmos (Table 1).

The histological study of the thyroid glands of the controls revealed general hypertrophy and hyperplasia with large cells and little colloid, as frequently encountered in hatchery-reared fish. Mitoses were abundant. In some animals masses of thyroid cells with rare and small colloid accumulations were invading surrounding tissues, such as bone marrow, derma, etc. The hyperplasia and hypertrophy were often more extreme than those observed here in laboratory animals with a high degree of iodine deficiency (rats and mice). In contrast, the animals given thyroid preparations had glands with very small cells and dense colloid.

Iodide treatment. The *growth* rate of parr living in water to which iodide was added appeared to be less than that of the controls (Table 2).

The *pigmentation* of the iodide-treated animals was moderately reduced at the end of this experiment (Table 2). There was little difference between the two iodide groups, except for slightly less pigmentation in the fish kept in water with 60 p.p.m. of added iodide.

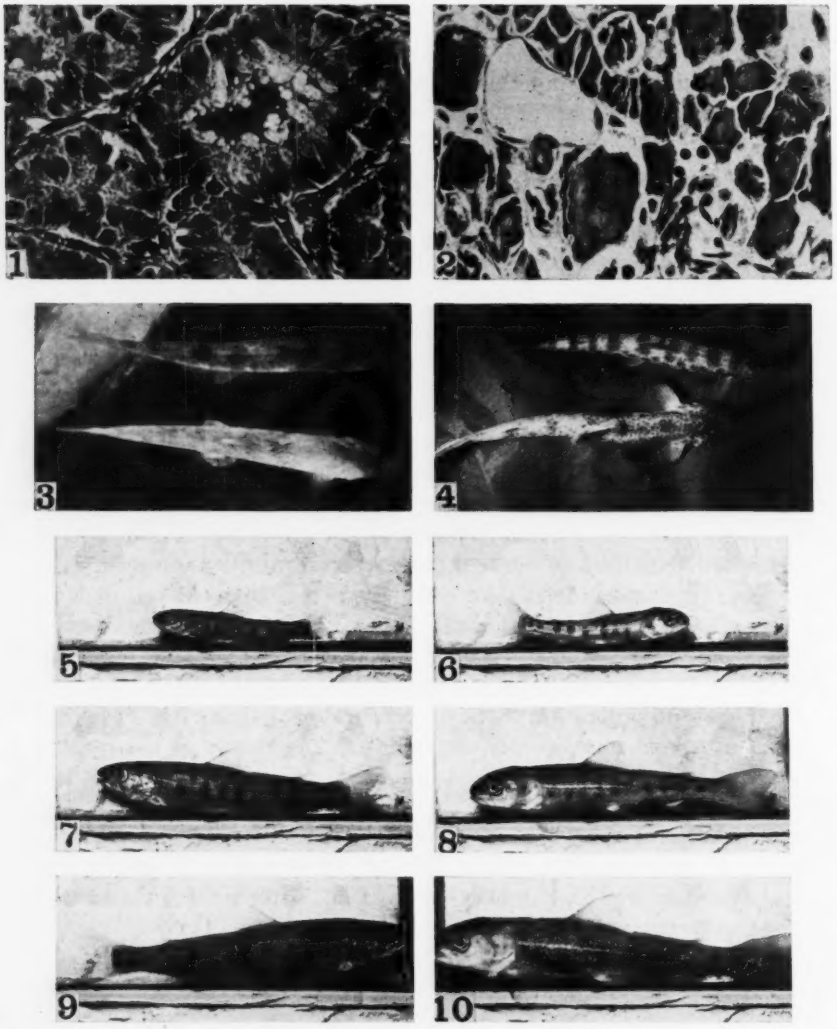

PLATE 1

EXPLANATION OF FIGURES

First series:

FIG. 1. Thyroid follicles of control salmon parr (fourth experiment) Masson's trichrome. High dry objective. ×350.

FIG. 2. Thyroid follicles of corresponding salmon parr living in water containing iodide in a concentration of 10 parts per million. Same stain and magnification as

The histological study of the thyroid glands of the controls showed a picture of hyperactivity with high cells and little colloid (Fig. 1) and invasive properties of the thyroid tissue, as described in other control groups of this series. The glands of the iodide-treated animals had smaller cells and a somewhat denser colloid (Fig. 2), thus giving a picture more comparable to that observed in normal thyroid glands of laboratory animals (rats and mice). The invasive features of the thyroid tissue were completely suppressed by the iodide treatment.

Second Series

In this series, thyroid feeding of salmon parr of various sizes was prolonged for a period of 5 months.

There was no significant effect on the *growth* in length of the parr, but the weekly increase in body weight was much reduced in the largest animals (Table 3).

In all subgroups, the *pigmentation* of the treated animals was less than that of the controls. The decrease in pigmentation was slight in the group of small parr (Figs. 5 and 6), moderate in middle size parr (Figs. 7 and 8)

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above. ×350. Note the difference in the size of the cells and the relative amount of colloid.

FIG. 3. Top view of live control (above) and thyroid-treated (below) salmon parr (third experiment). ×0.60. The treated parr is less pigmented than the control. The opercula of the treated protrude more than those of the control.

Second series (live animals):

FIG. 4. Top view of large-sized control (above) and thyroid-treated (below) salmon parr. ×0.30. The dorsal bars are visible in the control but not in the treated. The opercular protrusion is prominent in the treated.

FIG. 5. Side view of a small-sized control parr. ×0.40. The animal is well pigmented. The contour of the head is smooth.

FIG. 6. Side view of a small-sized parr treated with beef thyroid. ×0.40. The animal looks slightly paler than the control (Figure 5), especially in the ventral region, but the lateral bars are still visible. The contour of the head shows a protrusion on the top of the skull.

FIG. 7. Side view of a medium-sized control parr. ×0.40.

FIG. 8. Side view of a medium-sized parr treated with beef thyroid. ×0.40. The lateral bars are less visible than in the control (Figure 7) and also than in the small-sized treated parr (Figure 6). The head appears enlarged with a protrusion on top.

FIG. 9. Side view of a large-sized control parr. ×0.40. The caudal fin is transparent and V-shaped.

FIG. 10. Side view of a large-sized parr treated with beef thyroid. ×0.40. The pigmentation is reduced as compared with the control (Figure 9) and the lateral bars are barely visible. The head appears large. The fins are slightly more opaque than in the control, particularly the caudal fin, which is thicker and U-shaped.

and clearcut in the large parr, in which the lateral bars had faded more or less completely (Figs. 9 and 10).

The treated fish of every subgroup also showed *other changes*, including a progressive increase in the size of the head, noticeable 4 weeks after the treatment had started, as well as a protrusion of the opercula, visible in fish examined from above (Fig. 4) and protrusions on the top of the head, visible in side view (Figs. 6, 8 and 10). Upon close examination, the treated animals seemed to be covered by a grayish film, so that the head appeared less shiny than in controls (compare Figs. 7 and 8). Similarly, the fins appeared grayish and less transparent (compare tail fins in Figs. 9 and 10). These various changes were most pronounced in the large animals.

In an attempt to find out the cause of these transformations, the skin and other tissues were examined histologically. It was first thought that the grayish appearance of the fins was due to an impregnation of the connective tissue with calcium salts. However, sections stained by the von Kossa method showed no calcium salts in this location, nor was there any difference in the degree of calcification of bony tissues of control and treated animals. On the other hand, the skin of the treated animals showed a marked increase in the thickness of the derma (Figs. 11 and 12). This thickening was noticeable 5 weeks after the beginning of treatment, but was most pronounced in the animals treated for 5 months. The dermal increase was a true hyperplasia with increase in the number of both connective

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EXPLANATION OF FIGURES

Second series:

FIG. 11. Cross section of the head (dorsal surface) of control parr fed beef liver. Masson's trichrome. ×75. From top to bottom, note the dark staining epidermis, the light staining derma, bone, cartilage, meninges, and nervous system.

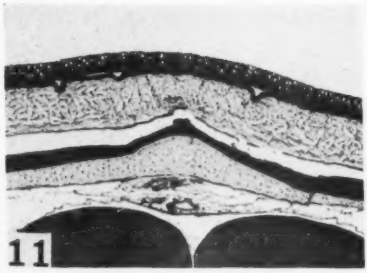
FIG. 12. Cross section of the head (dorsal surface) of a parr treated with beef thyroid. Masson's trichrome. ×75. The thickness of the derma is almost twice that of the control.

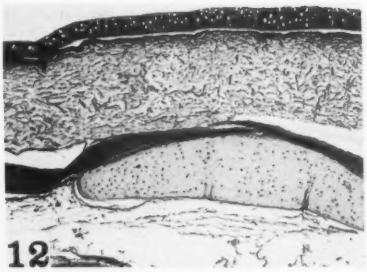
FIG. 13. Cross section of the eye of a control parr:—R: anterior edge of retina (*ora serrata*); L: palpebral fold of skin surrounding the visible portion of the eyeball. The cornea may be seen below. Masson's trichrome. ×75. Note the relative position of the retina and the palpebral fold.

FIG. 14. Cross section of the eye of a thyroid treated parr. Masson's trichrome. ×75. Note the change in the relative position of retina (R) and palpebral fold (L). The thickness of the derma is increased as compared with the control (Figure 17).

FIG. 15. Cross section through the upper jaw of control parr. Von Kossa stain, hematoxylin-eosin. ×75. From left to right, it is possible to see the epidermis and the derma, muscles with bone (black), lamina propria and epithelium of mouth.

FIG. 16. Cross section through the upper jaw of thyroid treated parr. Von Kossa stain. Hematoxylin-eosin. ×75. The derma is markedly enlarged, while the connective tissue underlying the mouth epithelium seems little changed.





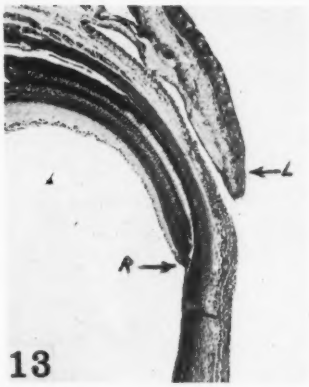


PLATE 2

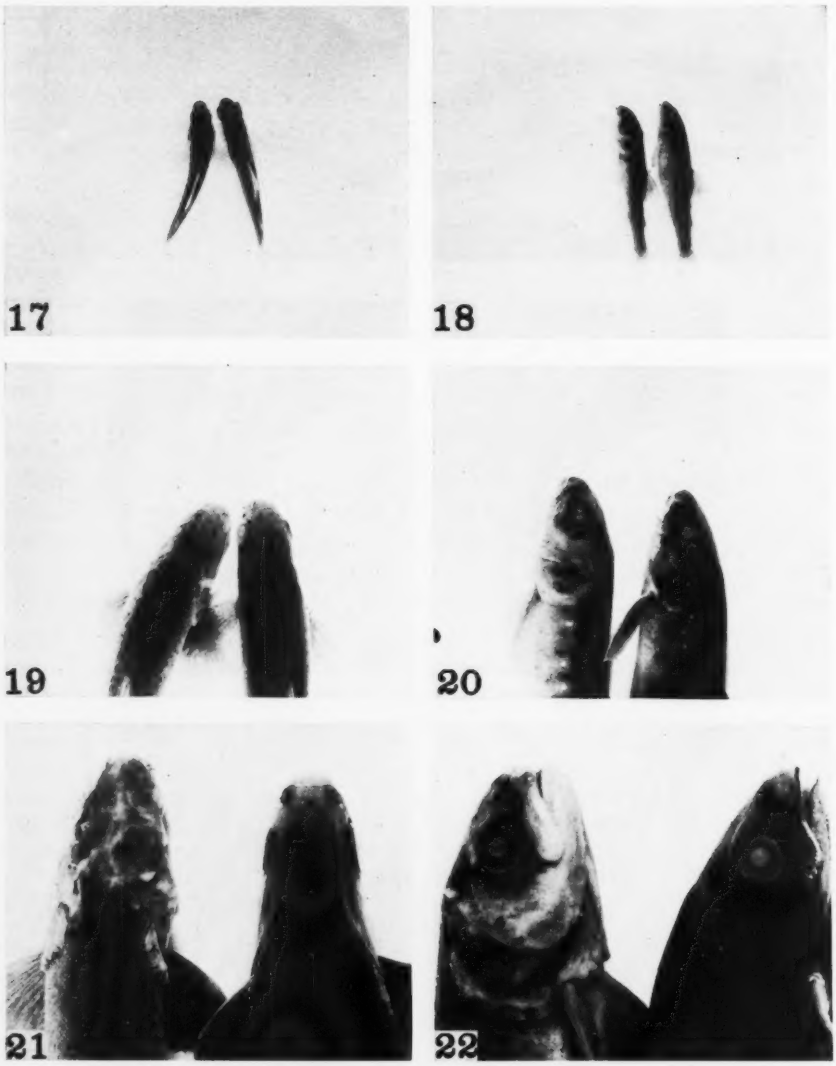


PLATE 3

EXPLANATION OF FIGURES

Third series:

FIG. 17. Dorsal view of a fry treated with beef thyroid on the left and a control fry on the right. ×0.60. The enophthalmos and protrusions on the opercula of the treated fry are visible.

tissue cells and collagenous fibers, without apparent change in their shape or staining properties.

The dermal thickening also explained why the palpebral fold of skin surrounding the eye was enlarged after treatment and extended further over the cornea than in control animals (Figs. 13 and 14, L). The thickening of the derma was especially evident around the edges of various structures, such as the edges of the mouth cavity (Figs. 15 and 16), the upper edge of the caudal fin, and the posterior and ventral edges of the opercula.

The connective tissue adjacent to the internal organs did not show the obvious signs of hyperplasia seen in the derma. Thus, in Figs. 15 and 16, the left hand sides of the pictures showed the hypertrophy of the dermal connective tissue, while on the right hand sides there was little or no difference between the mucosal connective tissue of control and treated animals.

Finally, there was some indication that the epidermis was increased in thickness too. This was definite in the animals sacrificed after a 5 month treatment (Figs. 13–16).

The histological study of the thyroid gland in controls showed follicles lined by cuboidal cells and containing a fairly large amount of colloid—a picture comparable to the "normal" thyroid of laboratory animals, but quite different from that in the controls of the first series. The cause of the difference between these two control groups is not known. The effect of thyroid treatment however, was the same as in the first series, as the cells became very small and the colloid dense.

Third Series:

This series included salmon fry, parr, and smolt as well as trout parr.

The body weight of the fry was not affected by thyroid treatment, while treated parr gained somewhat more weight than the controls (Table 4).

FIG. 18. Lateral view of a fry treated with beef thyroid on the left and a control fry on the right. ×0.60. Protrusion on top of the head in the treated fry.

FIG. 19. Dorsal view of a parr treated with beef thyroid on the left and a control on the right. ×0.60. Moderate depigmentation and enophthalmos of the treated animal.

FIG. 20. Lateral view of a parr treated with beef thyroid on the left and a control on the right. ×0.60. Moderate depigmentation, especially visible below the eye and in the ventral region, and enlargement of the operculum in the treated animal.

FIG. 21. Dorsal view of a smolt treated with beef thyroid on the left and a control smolt on the right. ×0.60. The skin of the head of the treated smolt is thrown into irregular folds, as far back as the posterior edge of the operculum which appears ruffled, while in the control the edge is smooth. The treated smolt is also less pigmented than the control.

FIG. 22. Lateral view of a smolt treated with beef thyroid on the left and a control smolt on the right. ×0.60. The thickening of the skin, especially around the operculum is very striking in the treated. The pigmentation is less in the treated than in the control.

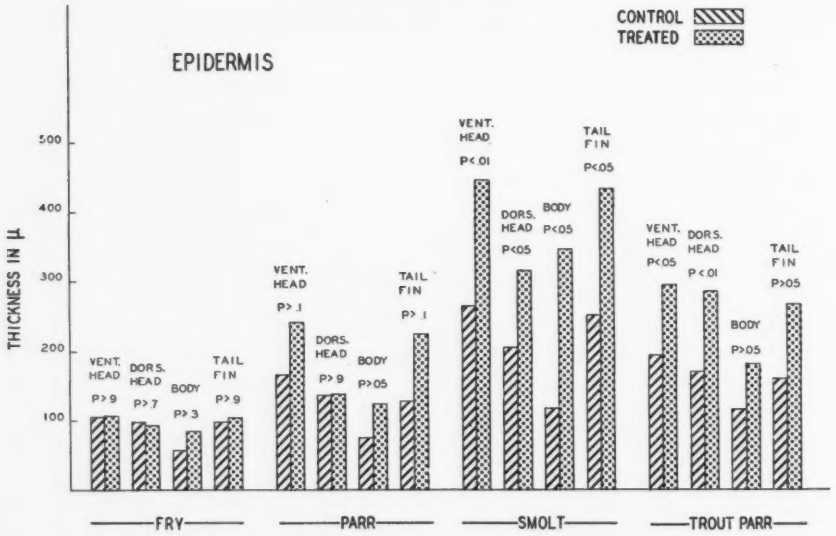


FIG. 23. Thickness of the epidermis in control and thyroid-treated animals of the third series. Four areas of the integument were examined: the ventral region of the head (Vent. Head), the dorsal region of the head (Dors. Head), the region of the body between dorsal and adipose fins (Body), and the anterior portion of the tail fin (Tail Fin). The results of statistical analysis are placed above the paired columns as P values. Values lower than .05 were considered significant.

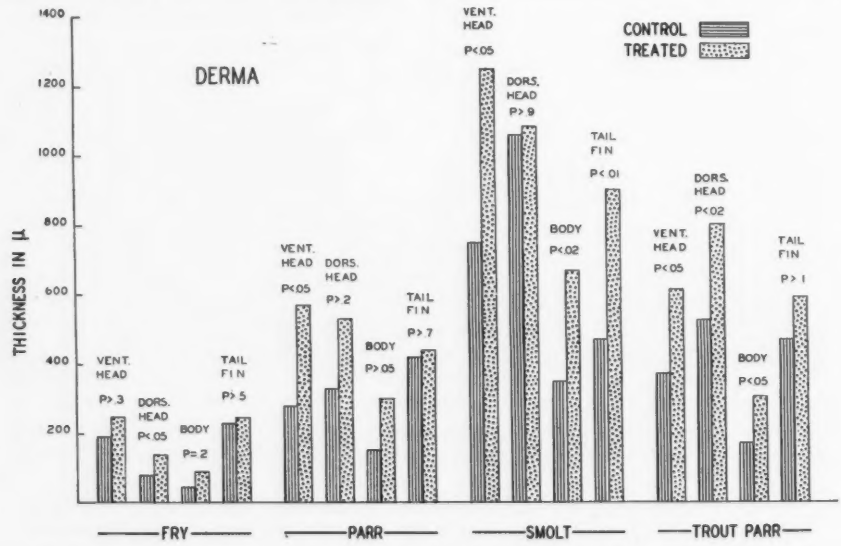


FIG. 24. Thickness of the derma in control and thyroid-treated animals of the third series. Abbreviations as in Figure 23.

The growth of treated smolt was definitely retarded. They looked emaciated and their weekly increase in body weight was much less than in controls (Table 4). No changes in the growth rate of the treated trout was observed.

The pigmentation of treated fry was slightly reduced (Figs. 17 and 18). The pallor was more pronounced in treated parr, whether dorsal or ventral surfaces were considered (Figs. 19 and 20). The effect occurred earliest and was maximal in smolt (Figs. 21 and 22). The treated trout also showed a marked depigmentation at the end of the experiment.

The number of pigment cells was counted per unit area in both ventral and dorsal skin of treated and control fry, parr and smolt. The results (Table 4) demonstrated a statistically significant decrease in the number of pigment cells in the ventral region of all treated groups, as well as in the dorsal region of the parr.

Finally, to further our understanding of the significance of the depigmentation, the integument was scraped off with a scalpel in several areas, particularly in the region of the lateral bars (parr and smolt) to expose the deeper pigment cells. The pigment cells of the lateral bars, barely visible in the treated fish, became quite distinct after removal of the skin. But even when treated and untreated animals with skin removed were compared, the treated appeared lighter than controls.

Other changes due to thyroid treatment were again visible, particularly the thickening of the derma in treated fish. This was slight in fry, but definite in parr. The various features listed above, grayish appearance of the skin, protrusion of the opercula, enophthalmos, etc., were definite in parr. In the smolt the thickening of the skin was particularly obvious, with thick folds of this tissue appearing on the head (Figs. 21 and 22; see also Figs. 26 and 27).

Histometric measurements revealed that thyroid treatment produced a thickening of the epidermis which was statistically significant in all regions examined in the smolt and in all but the ventral region of the head in the trout parr. Whatever thickening of the epidermis was observed in treated salmon fry and parr was not statistically significant (Fig. 23).

In the case of the derma (Fig. 24), the treated fry had a significantly thicker derma in the dorsal region of the head than their respective controls. In treated parr, the increase was significant in the ventral region of the head. The treated smolt exhibited a significant thickening of the derma in all regions examined, except in the dorsal portion of the head. The treated trout parr showed a significant increase in the derma in all areas but the tail fin. Microscopical examination of the derma of treated fish (Figs. 26 and 27) showed that all elements of the connective tissue including the fibroblast nuclei had increased in number, but their size did not seem to have been affected.

The thickness of the whole skin was examined by adding up the figures

for the thickness of epidermis and derma (Fig. 25). The control data, as summarized by the broken line, suggested that the skin grows in proportion to the size of the animal at the fry and parr stages, while at the smolt stage an upward swing of the growth curve reveals a considerable increase in the development of the skin. Thyroid treatment shifts the curve to a higher level, indicating a thicker skin in all groups (solid line; Fig. 25),

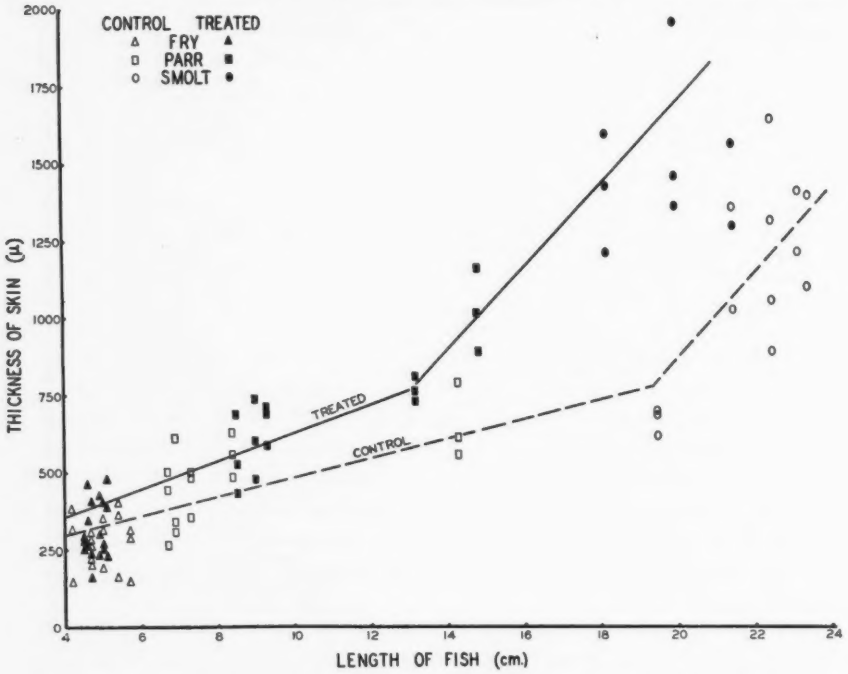


FIG. 25. Over-all thickness of the integument plotted against body length in control (light symbols) and thyroid-treated (dark symbols) animals of the third series. The lines drawn—a broken line for the controls and a continuous line for the treated animals—represent a somewhat speculative interpretation of the data indicating the growth curve of the skin.

and, furthermore, produces an earlier upward swing of the growth curve due to a considerable increase in the thickness of the skin not only in smolt, but also in large parr. Thus, after thyroid feeding, the size of the skin in the largest of the treated parr was comparable to that in control smolt.

Finally, thyroid treatment also produced an enlargement of the head which was slight in salmon, but considerable in trout. The head was much broader after treatment in this species (Figs. 28 and 29). That the control

in Fig. 28 closely matched the treated animal in Fig. 29 may be seen by the similarity of the size of the nervous system, eyeball, optic nerve, and muscle masses in both cases. In contrast, there was a pronounced difference in the distance between the two eyeballs. The average interorbital distance was 2.05 mm. in the controls and 4.95 mm. in the treated. Furthermore, the amount of connective tissue present in the orbital space was much greater in treated (Fig. 28) than in control (Fig. 29) animals. This accumulation of connective tissue may be responsible for the change in the shape of the head.

The histological study of the thyroid glands gave the same results as in the first series, that is, a hyperactive picture in the controls and a completely resting picture in the thyroid treated animals.

DISCUSSION

Effects of thyroid extracts in salmonidae

An extreme resting condition of the thyroid gland was observed in all young salmonidae given thyroid extracts or thyroxine, regardless of the original condition of the gland. This effect, which is identical to the well known effect of thyroid preparation in mammals, may be taken as a proof of the passage of thyroid-active material into the circulation. That this material had a systemic effect in young salmonidae was proved by 3 types of observations.

The first finding was that there was some retardation of *growth*. More precisely, thyroid treatment slowed down the increase in body weight, but the increase in body length was not affected. This was clearcut in treated smolt, which gained weight more slowly than their controls (Table 4) and looked emaciated on gross examination. Treated parr also gained weight more slowly than their controls, but this effect was definite in only the largest of these animals (Table 3).

The second and most significant effect of thyroid administration *was a thickening of the layers of the integument.* In treated smolt, a gelatinous swelling of the skin was evident on gross examination, particularly on the edges of the opercula (Figs. 21 and 22). In treated parr, the changes were less obvious, but tails and fins had a swollen, grayish appearance (Fig. 10).

Histologically, the skin of thyroid treated fish usually showed an enlargement of both epidermis[2] (Fig. 23) and derma (Fig. 24). The increase in the derma appeared first and may, therefore, have produced a distension of the epidermis, which resulted in the hyperplasia of the latter. The thick-

[2] In contrast, thyroxine produced a thinning of the epidermis in rats (Eartly *et al.*, 1952). The hormone in this case was administered in physiological doses to thyroidectomized rats.

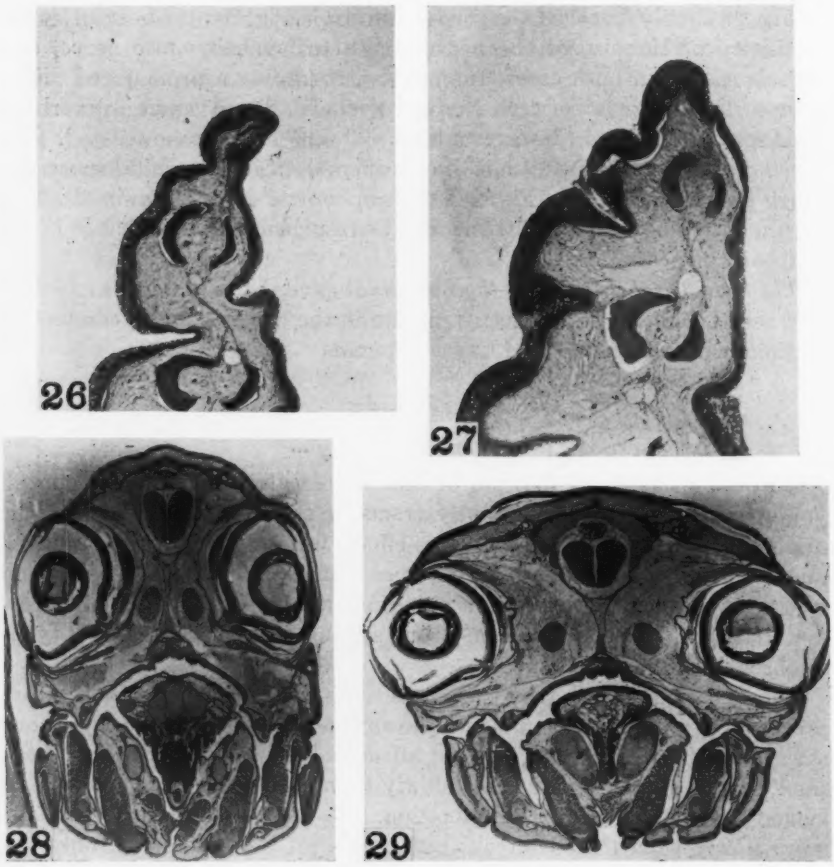


PLATE 4

EXPLANATION OF FIGURES

Third series:

FIG. 26. Cross section of the pectoral fin of a control smolt fed beef liver (third experiment). Hematoxylin-eosin. ×70. Epidermis, derma and fin rays may be identified.

FIG. 27. Cross section of the pectoral fin of a smolt treated with beef thyroid. Hematoxylin-eosin. ×70. The epidermis has increased in thickness, at least on the dorsal side of the fin (left of the picture). The derma also shows an increase on the dorsal side (left).

FIG. 28. Cross section of the head of a control trout parr (fourth experiment) at the level of the eyeballs. ×5.3.

FIG. 29. Cross section of the head of a thyroid treated trout parr at the level of the eyeballs. ×5.3. A considerable increase in the size and content of the space between the eyeballs may be noted in this animal as compared with the control (Figure 28). There is

ening of the derma consisted of an increase in the number of fibroblasts and collagenous fibers without change in their size, organization or staining reactions.

While the increase in the thickness of the derma after thyroid treatment was not statistically significant in all areas under examination (Fig. 24), the trend towards such an increase was generally present. The hyperplasia was especially prominent wherever there was a fold of skin (Figs. 14 and 16). However, connective tissue other than that of the derma did not respond to thyroid treatment in young salmon. In treated trout, an increase was found in the interorbital distance, presumably as a result of the increase in the amount of loose connective tissue between the eyeballs (Figs. 28 and 29). Since in this area the connective tissue mainly consisted of ground substance with few cells or fibers, the thyroid treatment must have augmented the amount of ground substance. It was pointed out above that, in other areas, the number of fibroblasts and collagenous fibers was greater after treatment. It may, therefore, be concluded that the ground substance, as well as fibroblasts and collagen—that is, the three main elements of connective tissue—increase in amount in thyroid-treated fish.

The enophthalmos of treated animals (Fig. 3) was not due to a decrease in the distance between the two eyeballs (in fact, this distance was increased in trout; Figs. 28 and 29), but rather to a thickening of the integument in which the eyes were buried. Histologically, the palpebral fold surrounding the eye showed some extension as a result of the dermal hypertrophy and thus partly covered the eyeball (Figs. 13 and 14). The enophthalmos was, therefore, apparent, not real. The lateral protrusion of the opercula (Figs. 3 and 4) as well as the protrusions on top of the head (Fig. 8) might also be due to accumulation of connective tissue. In guppies the effects of thyroid extract on the shape of the fins (Krokert, 1936) and the increased differentiation rate of the gonopod (Hopper, 1950, 1952) might be the result of the thickening of dermal connective tissue. The similar effects on the fin growth and gonopod differentiation observed in Platypoecilus, may also be explained by an extensive development of the connective tissue (Grobstein and Bellamy, 1939). At any rate, the most striking change attributable to the effect of thyroid treatment on the connective tissue was the modification in the shape of the trout head (Figs. 28 and 29).

The third effect of thyroid treatment was the *integumentary pallor* already reported by Landgrebe (1941), Robertson (1949) and La Roche *et al.* (1949, 1950). Here, this phenomenon was observed mainly in older

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also an increase in the thickness of the epidermis and derma (see top). The size of most other structures is identical to that in the control animal. The hyperplasia of the interorbital connective tissue may partly explain the change in the shape of the head.

salmon parr (Figs. 3–10), salmon smolt (Figs. 21 and 22) as well as in brook trout parr. Robertson (1949) described 3 modifications of the pigment cells which would account for the pallor of treated trout, namely, decrease in number, contraction, and covering of these cells by opaque guanine deposits on the inner surface of the scales. In the present work, a moderate but statistically significant decrease in the number of pigment cells per unit area was usually present, particularly in the ventral region (Table 4) where the difference in cell number between control and treated could often be recognized with the naked eye.

Contraction of pigment cells could play only a minor role in the pallor of treated animals, since the contrast between the dark control and the light treated parr persisted even after the expansion of pigment cells by urethane anesthesia. Neither could the deposition of guanine on the inner surface of the scales be a major factor, since the pallor of the treated animals was just as definite in the scaleless head region (Figs. 21 and 22) as in the rest of the body where scales are abundant.

On the other hand, the thickened skin and particularly the thickened derma formed a grayish film at the surface of the body, thus covering and partially hiding the deeper layer of pigment cells. This mechanism may play a role in the fading of lateral bars in large treated parr, for after removal of the derma so as to expose the deep pigment cells, the lateral bars became quite distinct in treated animals, although they remained lighter than in controls. Briefly then, the pallor of the thyroid-treated fish was mainly due to a moderate decrease in the number of pigment cells, while the increase in dermal thickness may also have obscured the deeper layer of melanophores.

In conclusion, objective measurements demonstrated that the main effects of thyroid treatment in young salmonidae were an increase in the size of both skin layers, epidermis and derma, and a moderate decrease in the number of melanophores per unit area.

Role of the thyroid gland in the transformation of salmon parr into smolt

Histological signs of activity were observed in the thyroid gland at the time of the transformation of parr into smolt (Hoar, 1939). Hoar suggested that the thyroid gland may control smoltation in the same manner as this gland induces the metamorphosis of tadpoles into adult types of anurans. Fontaine (1948) presented various data in support of this conclusion. The present results, though they did not prove the validity of this hypothesis, did give it some support.

Smolt differ from parr by a larger size, by a more pronounced pallor (the so-called "silvering" of their ventral region) and—as shown here—by a

relatively thicker skin. That the skin thickens at about the time of the transformation of parr into smolt was shown by the growth curve of the skin, which exhibited an upward surge at the point where the animals had reached the smolt stage, as shown by the dotted line in Fig. 25. Thus, the change from parr to smolt would consist of a thickening of the integument in addition to the silvering.

The effect of thyroid treatment was also to produce pallor and thickening of the integument. The increase in skin thickness under treatment was particularly striking in older parr, since the upward surge of the skin growth curve occurred in these animals rather than at the smolt stage, as shown by the solid line of Fig. 25. Thus, after thyroid treatment, the thickness of the skin in the large parr was comparable to that in control smolt. In regard to the silvering, Landgrebe (1941), Robertson (1949) and La Roche *et al.* (1949) concluded that the pallor and fading of the lateral bars produced by thyroid-treatment of parr made them look like smolt.

However, the doses of thyroid extract employed here as well as by Landgrebe (1941) and Robertson (1949) were probably greater than the amounts of hormone normally secreted by the thyroid gland. And the thickening of the skin in the treated animals often reached a degree in excess of that found in nature (e.g., Fig. 22). Therefore, it would be desirable to see whether similar results could be obtained under more nearly physiological conditions.

In this connection it should be pointed out that hyperplasia and pallor of the skin were obtained not only with thyroid extracts, but also with thyroxine—which is presumably a hormone of the thyroid gland (Leblond, 1951). Furthermore, comparable results could be produced in parr by merely enriching their environment with iodide (Table 2). Under these conditions, the animals developed a pallor identical to, though less pronounced than that due to thyroid treatment. Since the control animals of this experiment had highly hyperactive thyroid glands (Fig. 1), they were presumed to be in a condition of iodine deficiency, and the output of hormone by their glands must have been very low. The administration of iodide had, therefore, provided the essential raw material for the elaboration of thyroxine by the gland. A higher level of circulating hormone should have ensued, resulting in dermal hyperplasia and pallor. It was concluded that secretion of thyroxine by the thyroid gland of the salmon parr itself might induce effects similar to those observed following administration of large doses of thyroxine or thyroid extract.

The similarity between the pallor and thickening of skin due to thyroid extract and the changes observed during the transformation of parr into smolt suggests that thyroid secretions may produce or at least facilitate the

occurrence of these changes.[3] Thus, the present experiments offer some support for the hypothesis of a thyroidal control of the transformation of parr into smolt.

SUMMARY

1. Various thyroid preparations, including thyroxine, were administered to Atlantic salmon, *Salmo salar*, at the fry, parr and smolt stages (and also to brook trout, *Salvelinus fontinalis*, at the parr stage). Iodide alone was given to parr salmon.

2. The main effect of thyroid preparations is to induce a pallor and a thickening of the integument, both of which are slight in fry, moderate in parr, and intense in smolt.

The pallor (Figs. 3–10) is due to a moderate decrease in the number of pigment cells per unit area and is augmented by the opacity of the thickened integument.

The increase in skin thickness involves both derma and epidermis. The dermal increase is the first to appear and may reach extreme proportions, in which case it is evident to the naked eye (Figs. 21 and 22). In trout, the effect extends to connective tissues other than that of the derma, particularly in the interorbital region (Figs. 28 and 29). The broadening of the head produced in this species may be a consequence of the hyperplasia of the interorbital connective tissue.

3. Administration of iodide to salmon parr (assumed to be in a state of iodine deficiency) produces a similar though less pronounced pallor than that due to thyroid extract. The extra iodide presumably provides the thyroid gland with the necessary raw material for the synthesis of sufficient thyroxine to induce the pallor of the integument.

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[3] A sequence of iodine deficiency and abundance may occur in salmon parr as they reach estuaries.

In general, the water of the rivers of cold countries in which parr live contain much less iodine than those of warm areas. Thus, it was calculated from the data of McClendon and Hathaway (1924) that, in the United States, the average iodine content was much lower in the water of the northern rivers (0.00008 parts per million and even 0.00001 p.p.m. for the water of the salmon rivers of Oregon) than in that of the southern rivers (0.00193 p.p.m.). On the other hand, Fellenberg (1925) showed that river water (0.00056 p.p.m. for the average of samples from 12 Swiss rivers) contained much less iodine than sea water (0.023 p.p.m.). Parr coming down a river to the brackish water of an estuary are first exposed to a low then to a high concentration of iodine. They are, therefore, first subjected to a condition of iodine deficiency and then to one of abundance. Consequently, the output of hormone by the thyroid glands may be increased as they reach the estuary resulting in an effect similar to that of thyroid treatment. Indeed the transformation from parr to smolt, in some, but not all, of the areas where *Salmo salar* lives, takes place at or near estuaries, that is, when the parr reach the iodine-rich water.

4. The thyroid hormone may play a role in the increase in pallor and thickness of the integument which occurs under natural conditions when salmon parr transform into smolt.

Acknowledgement

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METABOLISM OF RADIOIODINE BY THE NEAR-TERM BOVINE FETUS

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THE development of the thyroid gland in a placental organism presents certain highly interesting problems which invite investigation. More complete understanding of the interrelationship of the maternal thyroid-pituitary balance and that of the corresponding endocrine organs of the fetus seems especially worth while. That this interrelationship may be important appears to be indicated by the frequency of goiters during and after human pregnancy (Hiilesmaa, 1948). In studies of this problem the fetal bovine thyroid presents certain advantages because of its relatively large size. Indeed, recent analytical work by Wolff *et al.* (1949) and Koneff *et al.* (1949) has served to demonstrate that measurable amounts of organic iodine begin to accumulate in the thyroids of cattle as early as the sixtieth day of gestation (total gestation period of about 285 days). Furthermore, they have made the interesting observation that thyroxine is already present at this early stage, and that subsequently it increases in proportion to the growing concentration of total iodine in the fetal thyroid gland.

In beginning a study of biochemical differentiation in the fetal bovine thyroid, we elected to observe, by several different techniques, the fate of radioiodine administered to the cow in late pregnancy. The principal objective in this work was the isolation and characterization of the newly formed I^{131}-labelled fetal thyroglobulin, using the precipitation techniques described by Derrien (1947) modified for the purpose by Roche, Michel, Deltour, and Lissitzky (1951). By enzymatic hydrolysis of these thyroglobulins further information was sought concerning their proportions of iodinated amino-acids. Similar quantitative study of maternal blood and thyroid provided data relating to the partition between mother and fetus of administered I^{131}, and the occurrence of free iodinated amino acids. Finally, by radioautography of histological sections the microscopic distribution of newly metabolized I^{131} was studied in the maternal and fetal thyroids.

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MATERIALS AND METHODS

Preliminary treatment. Two gravid cows of "Normande" stock were injected intravenously with about 3.0 millicuries of carrier-free I^{131}, as iodide. An ox of "Monceau" stock was similarly treated. All were killed after an interval close to 24 hours after injection. Thyroid glands were dissected out immediately after death and small fragments were fixed in Bouin's solution for sectioning and radioautography. All unfixed tissues were frozen within two hours after collection. Blood samples were collected at the same time, adult blood being taken from the jugular vein, and fetal blood from the umbilical blood vessels. Both gravid cows had single embryos whose measurements are recorded in Table 1.

TABLE 1

Sex	Fetus No. 1	Fetus No. 2
	Female	Female
Weight, kilograms	23	50
Crown-rump length, cm.	69	
Chest circumference, cm.	60	
Abdominal circumference, cm.	58	
Weight of thyroid, grams	12.58	12.6
Maternal thyroid, grams	43.55	29.3

Preparation of Thyroglobulin. The frozen maternal and fetal thyroids were cut into thin slices (about 0.2–0.5 mm.) with razor blades. The slices were suspended in double their weight of 0.9% saline solution, and extracted at about 10° C. overnight. By centrifugation the saline extract was separated from the tissue residue and a re-extraction was made with one half volume of saline solution. The nitrogen contents and radioactivities of the combined first and second extracts were measured.

In working out the solubility curves of the I^{131}-labelled protein in the extracts, aliquots were mixed with ammonium sulphate solutions of concentrations from 30% to 60% under controlled conditions (Roche *et al.*, 1951), and the distribution of I^{131} and nitrogen in the supernatant and precipitate was determined. A relatively pure radioactive thyroglobulin was prepared from the precipitate coming down within a restricted range of ammmonium sulphate concentration, indicated by the first solubility curve to contain the largest part of I^{131}-labelled protein. This precipitate was redissolved and a second ammonium sulphate solubility curve was made (Fig. 1).

Hydrolysis and chromatographic analysis of radioactive thyroglobulins. The purified thyroglobulins were hydrolyzed by incubation with trypsin at pH 8.0 for 24 hours, at 38° C. Aliquots of the incubated material were acidified to approximately pH 1.0 with HCl and extracted with n-butanol. The concentrated butanol extracts were placed on sheets of Whatman #1 filter paper for one-dimensional, descending filter paper chromatography. In most instances chromatograms were prepared with two different solvents: n-butanol-acetic acid mixture or n-butanol-ammonia mixture. The distribution of radioactivity on the chromatogram strips was counted under an end-window Geiger tube, and radioautograms were prepared of the strips as well. Chromatogrammed in parallel with the hydrolysates as controls were monoiodotyrosine, diiodotyrosine, diiodothyronine, monoiodohistidine, thyroxine, and potassium iodide.

Determination of free iodinated amino acids in the maternal and fetal thyroidal extracts. A procedure similar to that suggested by Gross and Leblond (1951) was employed. Aliquots of the crude saline thyroidal extracts were extracted with 3 times their volume

of neutral normal butanol. Some acidified butanol extractions also were tried, and were more complete. After determination of their radioactivity these extracts were dried under vacuum at 45°–50° C. The residues were taken up in minimal volumes of butanol and one-dimensional filter paper chromatograms were prepared from them, using butanol-acetic acid as solvent in one series of tests and butanol-ammonia in others. The previously named six compounds were used as chromatographic controls, or as carriers in some instances. The chromatograms were scanned for distribution of radioactivity, and radioautographs were prepared as well.

Studies of maternal and fetal blood sera. Blood sera were collected by centrifugation of whole untreated blood samples. The "protein bound" or "trichloracetic acid-precipitable" I^{131} was determined. Two cc. volumes of the sera were precipitated with 5 cc. of 20% trichloracetic acid and centrifuged. The precipitate, after washing twice with additional

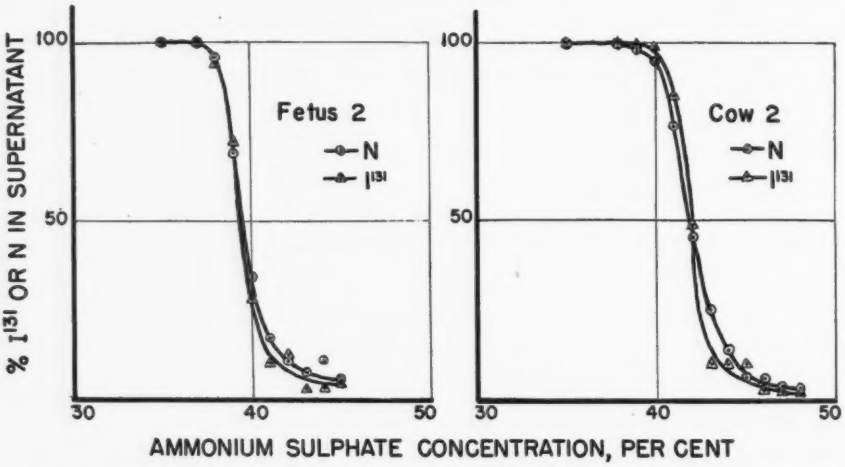

FIG. 1. Solubilities of purified I^{131}-labelled thyroglobulins of fetus No. 2 (left) and cow No. 2 (right) in ammonium sulphate solutions of different concentrations. Abscissa: concentrations of ammonium sulphate solutions as percents. Ordinate: percent of I^{131} (triangles) or nitrogen (circles) remaining in supernatant phase.

trichloracetic acid, was dissolved in 2N sodium hydroxide. The radioactivities of aliquots of the dissolved precipitate were measured and compared with previously determined measurements of the radioactivity of the whole serum. In another series of experiments, trichloracetic acid precipitates of blood serum were hydrolyzed with trypsin in a manner similar to that used for the thyroglobulin. Acid butanol extracts of these hydrolysates were analyzed by chromatography to determine their iodinated amino acid composition.

Radioautography of tissues. All fixed thyroidal tissue samples were sectioned after ordinary paraffin embedding. Before staining, the slides bearing the sections were used to prepare contact radioautographs, by exposure to No-screen X-ray films, or dental X-ray films. After autography the sections were stained with haematoxylin-eosin. By comparison of projected images of the sections and of their radioautographs, follicle by follicle presence or absence of storage of radioiodine was determined. It was concluded that since the tissues and sections had been passed through aqueous and alcoholic solu-

tions, xylol, melted paraffin, and alcohol-ether, that the radioiodine remaining for radioautography was most likely in protein-linked form, probably thyroglobulin.

RESULTS

Gross uptake of radioiodine by adult and fetal thyroids. The gross, unfractionated content of I^{131} in the thyroids was determined by counting the radioactivity in the crude saline extracts and in solutions of thyroid tissue "wet-ashed" in N NaOH. It is interesting that if the uptake of the mothers and fetuses are added together, they are comparable to the uptake by the single thyroid of the ox (19.7% and 22.9% as compared with 18.5% in the ox) (Table 2). On the other hand, it seems quite clear that in the presence of the smaller near-term fetal thyroids the maternal thyroids accumulated no more than a third as much as the ox. The concentration differences in I^{131} between mother and fetal thyroids is even more remarkable, being up to 7 times greater in the fetal gland.

TABLE 2. TOTAL THYROIDAL UPTAKES OF I^{131}, 24 HOURS AFTER INJECTION

	Per cents of dose of I^{131} injected			I^{131} Concentration ratio $\frac{\text{Fetus}}{\text{Cow}}$
	Thyroid of adult	Thyroid of fetus	Total	
Gravid Cow #1	7.55%	15.31%	22.86%	7.05
Gravid Cow #2	5.0	13.5	18.5	6.30
Ox	19.7		19.7	

Studies of thyroglobulins

a) *Ammonium sulphate solubility.* Curves of solubility of the I^{131}-labeled protein in ammonium sulphate solutions of increasing concentrations showed an unusually wide range of variation among the five tests. Though there are suggestions of differences between the adults and fetuses, the overlapping in ranges of ammonium sulphate solubility do not permit precise differentiation of an adult from a fetal type of thyroglobulin. The two fetal preparations contained the least soluble thyroglobulins; the two cow thyroglobulins seemed to overlap in solubility but also to be more soluble; the ox preparation was definitely the most soluble (Table 3, Fig. 1).

It should be noted, however, that in each case the thyroglobulin of the fetal thyroid was clearly less soluble than that of its own maternal thyroid.

b) *Radiochromatography of tryptic hydrolysates of the thyroglobulin preparations.* Acid butyl alcohol extracts of enzymatic hydrolysates of the purified thyroglobulins were analyzed by filter paper chromatography. Examination of Figure 2 shows that one important difference between the

TABLE 3. AMMONIUM SULPHATE SOLUBILITIES AND IODINE CONTENTS OF PURIFIED THYROGLOBULINS

	Range of precipitation, per cent $(NH_4)_2SO_4$ concentration	Per cent composition of stable iodine
Fetus No. 1	36–40	.78
Fetus No. 2	38–41	1.12
Cow No. 1	38–42	.58
Cow No. 2	40–44	.95
Ox	42–46	

adult and the fetuses is in the amount of thyroxine formed. Whereas both fetal thyroids formed significant quantities of I^{131}-labelled new thyroxine in the 24-hour post-injection period, no such newly formed thyroxine was demonstrable in the hydrolysates of the thyroglobulins of the ox and of one cow. The remaining cow formed an amount of thyroxine (about 5%), in this time interval, much smaller than was formed by its fetus. The only thyroidal hydrolysate in which free I^{131} (as iodide) was demonstrable by chromatography was that of a cow.

In terms of relative amounts of the radio-iodinated amino acids monoiodotyrosine and diiodotyrosine, no clear-cut distinction could be made between the fetal and adult thyroglobulins. In all hydrolysates but one approximately equal proportions of new diiodotyrosine and monoidotyrosine were found. In the one exception, a fetal preparation, a slight excess of monoiodotyrosine was found, despite the concomitant presence of new thyroxine (Fig. 2).

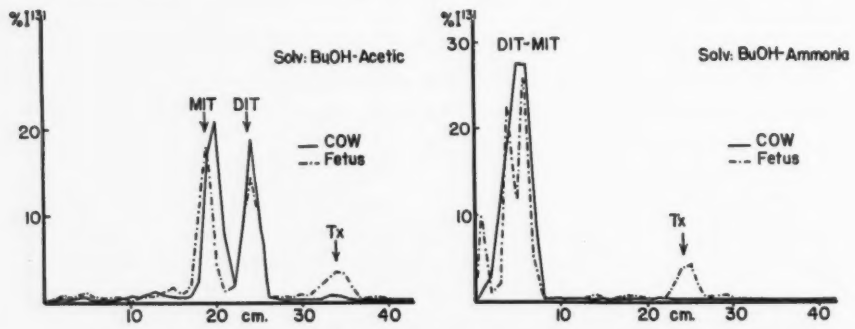

FIG. 2. Distribution of radioactivity along the length of filter paper chromatograms of trypsic digests of I^{131}-labelled thyroglobulins of a cow (solid lines) and its fetus (broken lines). The figure on the left illustrates this distribution when butanol-acetic acid was used as solvent. The figure on the right illustrates the result of using butanol-ammonia as solvent. Abscissa: length of the chromatogram in centimeters. Ordinate: per cent of the total radioactivity of the chromatogram. Symbols (positions of control substances): MIT, monoiodotyrosine; DIT, diiodotyrosine; Tx, thyroxine.

c) *Absolute thyroxine and I^{127} determinations.* Because of the observed absence of I^{131}-labelled thyroxine in chromatograms of the radioactive thyroglobulin of the first cow, a further effort was made to find radio-thyroxine and to determine the absolute amount of non-labelled thyroxine in this preparation. The thyroglobulin was hydrolyzed after the procedure of Blau (1933) with barium hydroxide. The hydrolysate was acidified and extracted with normal butanol at pH 4.0. The butanol extract was washed with sodium hydroxide and barium carbonate. The radioactivity of the butanol, or "thyroxine" fraction was measured, and found to be negligible. This confirmed the absence of radio-thyroxine as seen in the chromatographic analysis. The stable iodine of this same butyl fraction was then

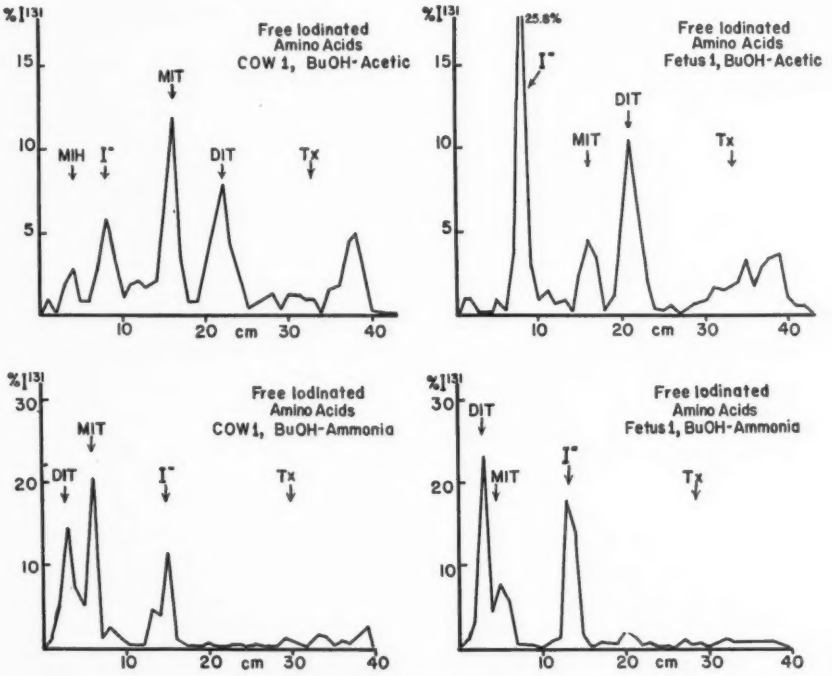

FIG. 3. Distribution of radioactivity along the length of filter paper chromatograms of simple butyl alcohol extracts of thyroid of the cow and fetus. Figures at the right show this distribution for extracts of thyroid of cow; figures at left show it for extracts of thyroid of the fetus. Upper two figures illustrate the distribution of radioactivity when butanol-acetic acid was used as solvent; lower two figures illustrate results when butanol-ammonia was used as solvent. Abscissa: length of chromatogram in centimeters. Ordinate: per cent of total radioactivity of the chromatogram found at any given point. Symbols (positions of control substances): MIH, monoiodohistidine; I, iodide; MIT. monoiodotyrosine; DIT, diiodotyrosine; Tx, thyroxine.

measured by the method of Leipert (1933). By this means it was determined that the "thyroxine" fraction contained 25.6% of the total iodine of the purified thyroglobulin of the cow. The total iodine content of this thyroglobulin, also determined by Leipert's method, was 0.58%.

The stable iodine contents of the other thyroglobulins were measured and are summarized in Table 3. It will be seen that the unusually high iodine content, 1.12%, in the thyroglobulin of fetus No. 2 is paralleled in the thyroglobulin of its mother, which contained 0.95% I^{127}. To our knowledge, these values, which were checked several times, are among the highest yet reported (Salter, 1940).

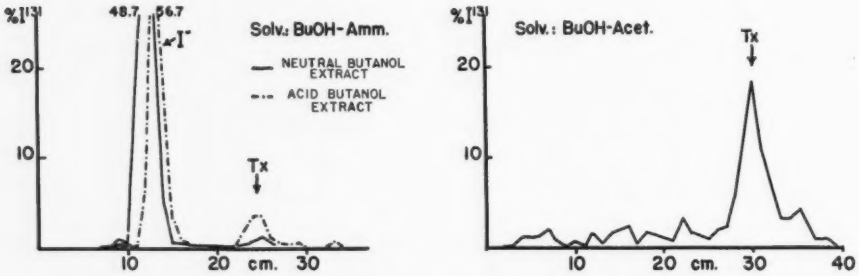

FIG. 4. Distribution of radioactivity along the length of filter paper chromatograms of (a) butanol extracts of fetal blood serum (left figure) and (b) of a trypsic hydrolysate of a trichoracetic acid precipitate of fetal blood serum (right figure). Abscissa: length of chromatogram in centimeters; Ordinate: per cent of the total radioactivity of the chromatogram found at any given point. Symbols (positions of control substances): I, iodide; Tx, thyroxine.

"Free," or butanol-extractable, iodinated amino acids in thyroid. Using Gross' and Leblond's (1951) procedure, or a slightly modified version (substitution of acidified butanol for neutral butanol for extraction) one dimensional radiochromatograms were prepared. As was noted by Gross and Leblond, the chromatograms obtained are complex and contain some "spots" which cannot as yet be identified (Fig. 3). We made no attempt to characterize any of the spots other than those which could be related to known compounds added to the extracts as carriers, or run parallel to them, or both. Definitely identifiable in the butyl extracts as "free" radioactive compounds were iodide, monoiodotyrosine and diiodotyrosine. No significant amounts of free thyroxine were found under the conditions of these experiments. In the series of chromatograms of one of the cows was found a spot agreeing with distribution of known monoiodohistidine (Fig. 3), both when butanol-ammonia was used as the developing solvent, as well as when butanol-acetic acid was used.

The extractable iodide, mono-, and diiodotyrosine, though present in both fetal and adult unhydrolyzed extracts, varied in proportion. It is clear that the fetal extracts contained a high proportion, and the maternal extracts a much lower proportion, of radio-iodide (Fig. 3, Table 4). The ox

TABLE 4. RELATIVE PROPORTIONS OF FREE IODINATED* AMINO ACIDS IN SIMPLE BUTANOL EXTRACTS OF CRUDE SALINE EXTRACTS OF THYROID TISSUE, EXPRESSED AS PER CENT OF TOTAL EXTRACTABLE RADIOACTIVITY

		Iodide	MIT	DIT	MIT+DIT	Tx
Fetus #1	BAC	32.6	10.3	25.4	35.7	dubious
	BAM	33.5	14.0	30.5	45.5	—
Fetus #2	BAC	58.5	12.3	14.2	26.5	dubious
	BAM	62.8	→	→	27.7	—
Cow #1	BAC	12.7	22.5	20.5	42.9	dubious
	BAM	20.3	27.3	26.4	53.7	—
Cow #2	BAC	18.1	32.2	37.6	69.8	—
	BAM	21.1	→	→	70.9	—
Ox	BAC	38.5	25.2	8.0	33.2	dubious
	BAM	41.8	→	→	43.2	(?)

* Iodinated with I^{131} at an interval 24 hours after administration of the isotope.
Abbreviations: BAC, Butanol-acetic acid; BAM, Butanol-ammonia; MIT, monoiodotyrosine; DIT, diiodotyrosine; Tx, Thyroxine.

butyl extract contained a high proportion of free iodide, being similar in this respect to the fetuses rather than to the cows (Fig. 5). The mono- and diiodotyrosines varied considerably in the different extracts, and not ac-

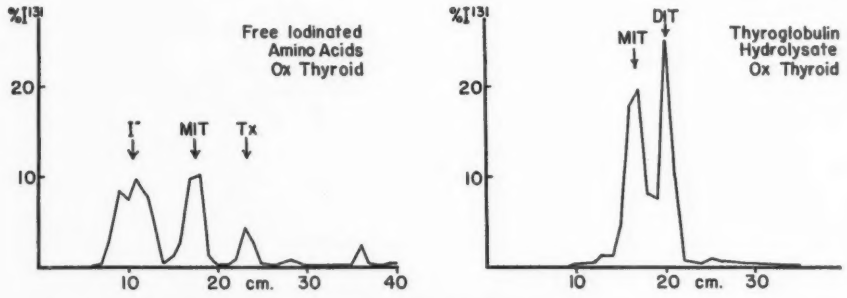

FIG. 5. Distribution of radioactivity along the length of filter paper chromatograms of (a) a simple butanol extract of ox thyroid (right figure); and (b) of a trypsic hydrolysate of the purified thyroglobulin prepared from ox thyroid (left figure). Abscissa, ordinate, and symbols as in previous figures.

cording to any recognizable pattern which could be correlated with their source.

Distribution of I^{131} in blood sera. The gross distribution of I^{131} in the sera of the cows and fetuses, at the interval of 24 hours after injection, was surprisingly equal considering the disparity in their thyroidal I^{131} concentrations. The blood concentration of I^{131} in the first cow was 73% of that of its fetus; in the second cow, 91% of that of its fetus. The trichloracetic acid precipitable, or "protein-bound," I^{131} in the first cow was only 40.2% of the total I^{131}, while the "PBI^{131}" of the fetus was 69.5%. The PBI^{131} of the ox was 77.2%.

At the time of sacrifice of the second cow, conditions prevented collection of a large enough maternal blood sample. For this reason a butanol extraction of whole serum could only be made for the fetus. This revealed, after chromatography, that most of the extractable radioactivity in the blood was due to free iodide and a small fraction to circulating radiothyroxine (Fig. 4).

Distribution of radioactivity in other organs. Samples of a number of organs of the injected animals were dissolved in 4N sodium hydroxide for measure of their radioactivity. In no case was the radioactivity significantly higher than that of the blood serum, indicating that at the 24-hour post-injection interval there was no significant accumulation of I^{131}. The organs examined were the following: salivary gland, ovary, tongue muscle, trachea, spleen, stomach, heart, lung.

Histology and radioautography of the maternal and fetal thyroid tissues. The morphology of the thyroids of the two cows was essentially similar. In both, the follicles were, as a rule, very large and lined by a low cuboidal epithelium (Fig. 6, 10a). The colloid, after hematoxylin-eosin coloration, was uniformly and densely eosinophilic. The colloid was hardly ever "vacuolated." Small nests of smaller follicles with a higher epithelium were infrequently encountered (Fig. 7). In general aspect, the maternal thyroids presented a histological picture of a low degree of function.

The thyroid of the ox was more active in appearance. The follicles were smaller in size than those of the pregnant cows, and the epithelial cells were cuboidal in shape. The colloid was more variable in type, varying from

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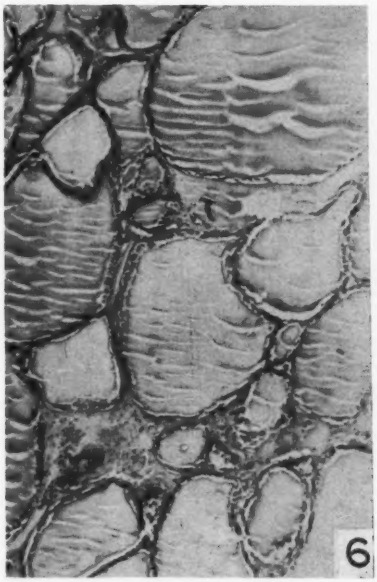
FIGS. 6–11. PHOTOMICROGRAPHS OF HISTOLOGICAL SECTIONS AND RADIOAUTOGRAPHS.

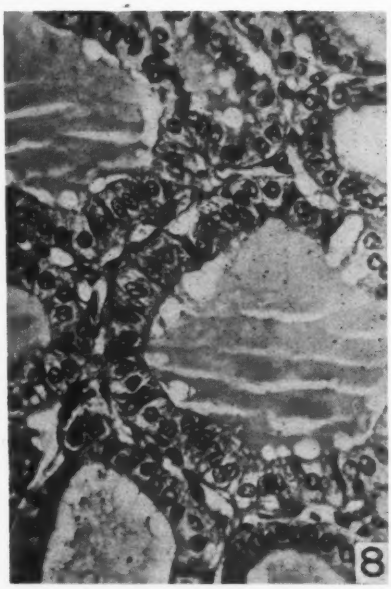
FIG. 6. Low magnification. Typical section of the thyroid gland of cow No. 1. Dense unvacuolated colloid, low epithelium, and large follicles.

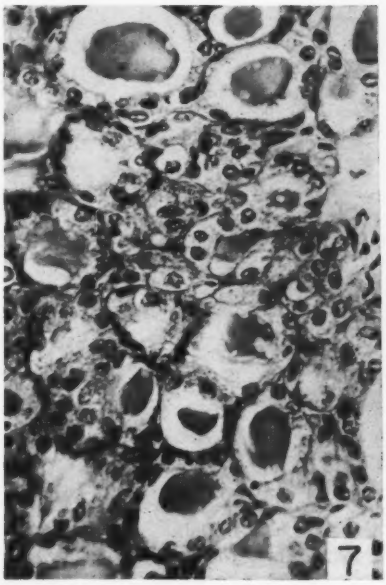
FIG. 7. High magnification of atypical small follicle area in thyroid of cow No. 1.

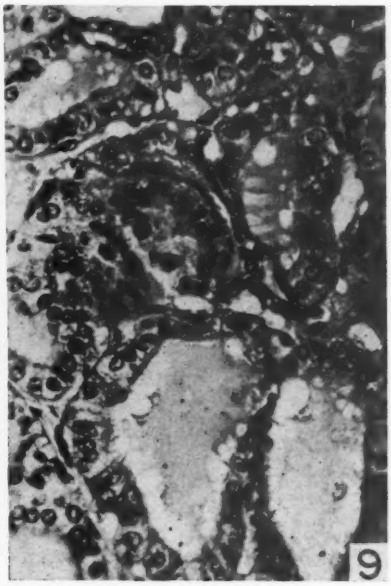
FIG. 8. High magnification of typical section of thyroid of fetus No. 1. Colloid less dense, and vacuolated at edges. Epithelium columnar, follicles uniformly smaller than in adult.

FIG. 9. Thyroid of fetus No. 1. Pycnotic area (middle and upper right-hand part of figure).









FIGS. 6–9

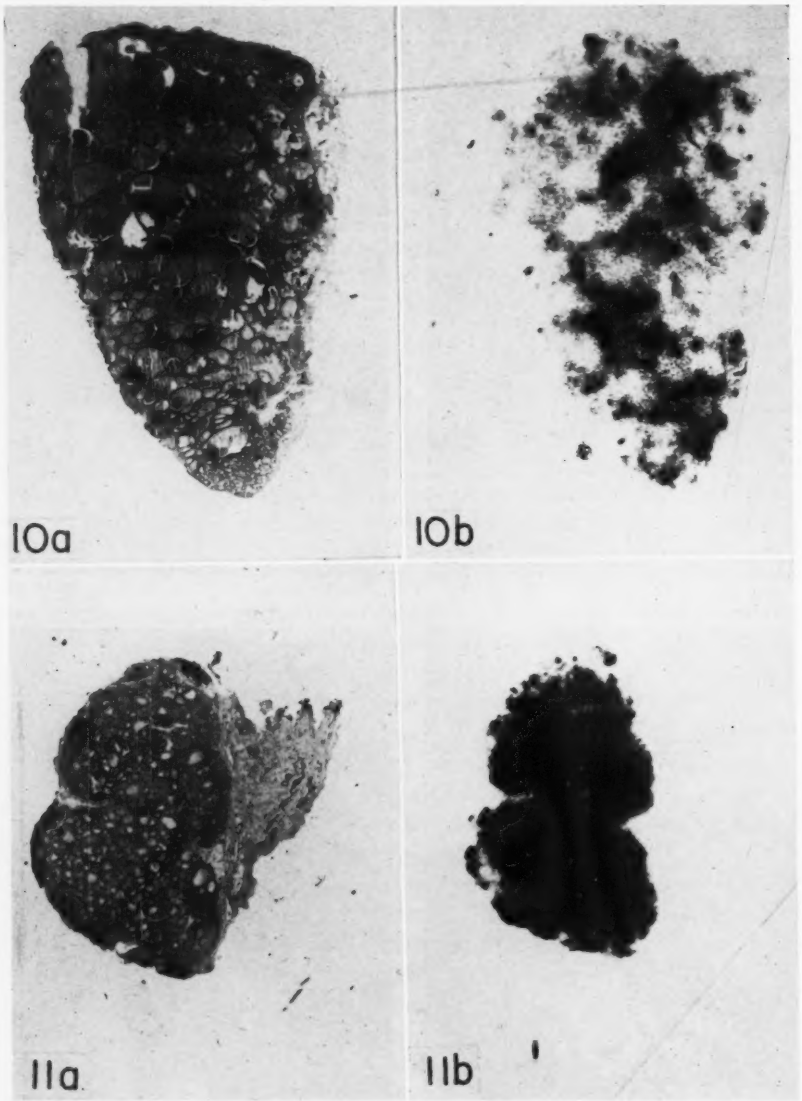


FIG. 10a. Very low magnification of a section of thyroid of cow No. 2; and figure 10b, the radioautograph made by the effect of this section on an X-ray film. Note the irregular distribution of I^{131} revealed by this technique. Most I^{131} is in the smaller follicles.

FIG. 11a. Very low magnification of a section of thyroid of fetus No. 2; and figure 11b, the radioautograph made by this section on X-ray film. Note the uniformity and greater intensity of I^{131} accumulation, in contrast with figure 10b.

densely eosinophilic to lightly eosinophilic. The latter type was usually vacuolated, and was found in follicles with a higher cuboidal epithelium.

The two fetal thyroids were the most active in morphological appearance of the series studied (Fig. 8). The follicles varied in size from very small in the medullary parts of the gland to large in the cortical regions (Fig. 11a). The epithelium varied in height in different follicles from cuboidal to columnar. Mitoses were only rarely seen, indicating that the proliferative process was not marked at this stage. The colloid was uniformly eosinophilic, and not much more vacuolated than the colloid of the ox. The fetal thyroids seemed to be much more vascular than those of the adults, and contained large lymphatic spaces. A peculiarity of the fetal thyroids was the presence of small centers of pycnotic degeneration (Fig. 9). These centers, as a rule, did not involve whole follicles, but adjacent epithelial portions of several different follicles. The cells involved in this process exhibited both swelling of nuclei and cytoplasm, and shrinkage and clumping of chromatin. These two degenerative phases were often contiguous. The pattern and distribution of these areas agrees well with that seen in the mouse after strong doses of I^{131} (Gorbman, 1950) and it is therefore assumed that this may be a radiotoxic effect in the fetal calf.

The radioautographs (Figs. 10, 11) of all thyroids studied indicated that the radioiodine present 24 hours after injection was located in the colloid. Differences noted between the different radioautographs were not only in their intensity, but also in distribution of the I^{131}. The fetal thyroids, as well as that of the ox, yielded much stronger autographs than did the maternal thyroids. This is, of course, a reflection of the greater concentration of radioiodine in the glands, as determined previously by quantitative techniques. The maternal thyroidal radioautographs, and those of the ox, in common, showed a discontinuity of distribution of isotopic iodine in the different parts of the gland. If the radioautographs can be taken as a picture of distribution of organically bound I^{131}, 24 hours after administration of the isotope, it is obvious that many follicles contained little or no radioiodine. These were usually the larger follicles with a flatter epithelium and a denser colloid (Fig. 6). In the fetal thyroids, on the other hand, the distribution of radioiodine among the follicles was uniform (Fig. 11).

DISCUSSION

The most striking findings in the present experiments are the extreme quantitative differences in iodine metabolism between the thyroids of the bovine mother and its near-term fetus. It seems remarkable that when the thyroid glands of mother and fetus have accessible to them at the same time a common pool of radioiodide, the small fetal gland accumulates more than twice as much as the large maternal gland after 24 hours of equilibra-

tion. The concentration differs by a factor of 7 in favor of the fetus. Furthermore, measurement of the radioactivity of the purified thyroglobulins reveals that the fetal protein contains more than 27 times the amount of isotopic iodine as does the maternal thyroglobulin. The presence of radiothyroxine in the fetal thyroglobulin and its absence or near-absence in the maternal hormone demonstrates the great difference in rate of formation of thyroxine in the two cases. This is all the more interesting when it is realized that the blood concentrations of iodine, 24 hours after administration of the tracer, are only slightly different, and that a large part of the fetal blood iodine is still in the form of iodide.

A number of possible explanations may be considered and, indeed, some which are susceptible to experimental analysis should be investigated for the data they promise toward the understanding of the well-known goitrogenic effect of pregnancy in humans.

First, it must be remembered that whereas the maternal system continuously is losing iodine by elimination through the urinary and digestive systems, the fetal system can lose iodine only if it is in a diffusible form, through the placenta. The conservation of administered tracer iodine would appear, therefore, to be better in the fetus than in the mother. It is, in fact, possible that in this way the fetus could act as a trap for maternal iodine, and that over a period of time the fetal system might rob that of the mother and exert a goitrogenic effect on the maternal thyroid.

The disparity in morphologic structure and rate of thyroxinogenesis between the maternal and fetal thyroids, if it can be considered to reveal a different systemic concentration of thyrotropic hormone, would seem to supply still another explanation for the functional differences observed. In fact, the histological inactivity of the maternal thyroids indicates that if the fetal thyroid is robbing the maternal system, it is not doing so to a degree sufficient to provide a stimulation to the maternal pituitary. This is seen not only in the histology, but also in the functional inability of the maternal thyroids to synthesize appreciable quantities of labelled thyroxine as late as 24 hours after I^{131} administration. In this sense the pregnant cow apparently is not different from non-pregnant cattle since the ox was similarly slow in thyroxine synthesis. Roche, Michel, Deltour, and Lissitzky (1951) have shown, furthermore, that only negligible quantities of radiothyroxine are formed when *in vitro* contact of ox thyroids is maintained with I^{131} under optimal conditions for 24 hours.

The possibility of robbing of the maternal system by that of the fetus may be considered from the opposite point of view. It is possible, for example, that the growing fetal thyroid forms enough diffusible thyroxine escaping into the maternal system, to act as an *anti-goitrogenic* influence, and that it may induce functional quiescence in the maternal thyroid. It is

known that thyroidectomized pregnant dogs show no signs of hypothyroidism until the birth of their pups, having been sustained meanwhile, apparently, by the thyroid secretion of the fetuses (Berblinger, cited by Guggisberg, 1936; and Salter, 1940).

The separateness of the thyrotropic supply of mother and fetus is demonstrated by such work as that of Jost (1949) who showed that hypophysectomy of the fetuses in rabbits results in arrested development and function of the fetal thyroids. This would indicate not only that the fetal hypophysis forms important quantities of thyrotropin, but also that circulating maternal thyrotropin is not readily available to the fetuses.

A disparity in thyrotropin secretion seems, therefore, a most attractive hypothesis to explain the difference in structure and function between the fetal and maternal thyroids, and even serves as a possible explanation of a reduction in function in the thyroid of the late-pregnant cow. A difference in reactivity of the fetal and maternal thyroids to equivalent concentrations of thyrotropin is another possibility, but is more difficult to assess.

The functional discontinuity seen in the radioautographs of the thyroids of the cows and the ox seems to be not unusual in adult glands since it also has been described in the rat (Leblond and Gross, 1949) and mouse (Gorbman, 1947). The uniformly active metabolism of I^{131} seen simultaneously in *all* follicles of the late fetal bovine thyroid would appear, therefore, to be a property of the young gland. The possible significance of these results in terms of distribution of the enzyme systems participating in thyroxine synthesis presents an interesting problem.

Without further study it would be difficult to know whether the observed differences in solubility of the fetal and adult thyroglobulins is consistent or significant. It is of interest, nevertheless, that in each of the mother-fetus pairs examined, the fetal thyroglobulin was definitely less soluble.

The iodine contents of the thyroglobulins in general agree with those of other mammalian species, or exceed them (Salter, 1940). The pair of values of 0.959% (cow) and 1.12% (fetus) are the highest values for thyroglobulin in our experience with mammalian thyroid.

In hydrolysates of the respective thyroglobulins, as noted before, little or no radiothyroxine was demonstrable for the adult thyroids (ox and cows). More than 20% of the radioactivity of the fetal thyroglobulin, on the other hand, was in the form of thyroxine. Because fractionation of the hydrolysates of the adult thyroglobulin revealed a composition of 25% non-radioactive thyroxine, it must be concluded that thyroxine synthesis in the adult bovine must be very slow. Since we know from the radioautographic evidence that most of the radioactivity was contained in the colloid of the adult thyroids examined, and since we know from the analytical

evidence that the radioactivity of the cow's thyroglobulin is mostly in the mono- and diiodotyrosine, the interesting question is raised as to whether or not the colloid itself can be the site of the final thyroxinogenesis. If not, the difficult assumption must be made that the incompletely metabolized compounds already bound to the colloidal protein, must be hydrolyzed off and reabsorbed into the epithelial cells for completion of the synthesis.

The butanol extractable, or "free," iodine and iodinated amino acids accounted for but a small fraction of the total radioactivity of the thyroid 24 hours after injection of the I^{131}, and it is difficult to assess their significance. Nevertheless, they varied in a regular fashion between the cow and fetus. The fetal glands contained a high proportion (30–60%) free iodide—as did the ox thyroid—in simple butyl extracts of fresh tissue. The cow thyroids both gave values of about 20% iodide. In no instance was thyroxine demonstrable in such extracts.

SUMMARY

Two near-term pregnant cows and one ox were injected with tracer quantities of radioiodine, and sacrificed 24 hours later. The distribution of the radioiodine was determined in the thyroids, blood serum, and various organs of the adult and fetal animals.

The blood sera of the fetuses contained only slightly more radioactivity than those of the cows, and in both this was in the form of iodide and a small proportion of thyroxine.

The fetal thyroids contained about twice as much I^{131} as the maternal thyroids. The concentration of I^{131} was six to seven times greater in the fetal thyroids. Other organs examined, including ovary and salivary gland, did not significantly concentrate I^{131}.

Purified thyroglobulin preparations were made from each of the thyroids by fractional precipitation in ammonium sulphate solutions under controlled conditions. Fetal thyroglobulin was 27 times more radioactive than that of the mother. In general, the fetal thyroglobulins tended to be less soluble than those of the adult animals. The stable iodine contents of the thyroglobulin preparations varied from 0.6% to 1.1%, each fetal thyroglobulin giving a value close to that of its mother.

Analysis of hydrolyzed thyroglobulins revealed that more than one-fifth of the radioactivity was due to newly formed thyroxine in the fetal protein, whereas maternal and ox thyroglobulin contained very little or no labelled thyroxine. This is due to slowness on the part of the adult thyroid to synthesize thyroxine, because measurement of the "stable" iodine in adult thyroglobulin hydrolysates revealed one-quarter of it to be in the thyroxine fraction.

Simple butanol extraction of the crude thyroid maternal for "free" iodine

and iodinated amino acids was made. In no case were there found more than questionable amounts of free thyroxine. The fetal thyroids, and that of the ox contained a much larger proportion of radioiodide than the thyroids of the two cows. Most of the butanol extractable radioiodine of the maternal thyroids was in the form of mono- and diiodotyrosine. In the thyroid of one cow identifiable monoiodohistidine was found.

Histologically, the maternal thyroids appeared much less active than those of the fetuses. Radioautographic localization of the radioiodine proved it to be in the colloid.

A disparity of thyrotropic hormone concentrations in the fetal and maternal circulation was considered the most reasonable explanation of the extreme disparity in thyroidal function between the mother and its near-term fetus.

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THE INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON THE METABOLISM OF THE THYROID HORMONE IN THE RAT*

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IT HAS been conclusively demonstrated that in many animals, including the rat, an increase in thyroid activity occurs on exposure to cold. (Ring, 1939; Uotila, 1939; Dempsey and Astwood, 1943). Uotila (1939) has shown that the pituitary stalk is a necessary link in the thyroid stimulation but beyond this, the mechanism of the response is obscure. In an attempt to elucidate further the manner in which exposure to cold causes an increase in the activity of the thyroid gland, we have studied the effect of environmental temperature on the serum protein-bound iodine first, in untreated rats, and second, in rats maintained on propylthiouracil and graded doses of thyroid. In the latter experiments, in which the rate of supply of thyroid hormone is known, one can obtain an index of the rate of utilization of thyroid hormone and can determine whether or not the increased utilization in rats exposed to cold is dependent upon an increase in the concentration of hormone in the blood stream.

METHODS

Male rats of a local Wistar strain with an initial average weight of 150 gm. were used. The animals were housed in individual cages in rooms equipped with a Bristol recording thermometer. The rats were fed Purina chow in dry, powdered form. They were weighed either daily or three times a week. The doses of U.S.P. thyroid were calculated on the basis of the weights obtained. The amount to be administered was mixed with a portion of the daily food small enough to be consumed entirely during the night. The rats were then given thyroid-free food ad libitum during the day. Propylthiouracil was given by mixing thoroughly one part of this drug with 1000 parts of the powdered food.

At the conclusion of each experiment the animals were anesthetized with 50 mgm. per kgm. of pentobarbital sodium given intraperitoneally. Following this as much

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blood as possible was withdrawn from the abdominal aorta with an oiled syringe and needle. The entire thyroid was then removed, trimmed free of extraneous tissue and weighed on a Taylor precision torsion balance. Finally half of the gland was weighed and placed in five per cent potassium hydroxide solution preparatory to determination of total iodine. The other half was fixed in Bouin's solution preparatory to histologic examination.

The protein-bound iodine of serum pooled from two to seven animals and the total iodine of thyroid glands pooled from the same animals were determined by the method of Man *et al.* (1942) or by a modification of the Barker chromic acid ashing method (Carr, 1950). In some experiments mean acinar cell heights were estimated by the method of Starr and Rawson (1937).

TABLE 1. NORMAL RATS EXPOSED TO WARM AND COLD ENVIRONMENTS

Exp. No.	Environmental temperature	Days of exposure	No. of animals		Mean daily weight gain, grams	Mean thyroid gland weight[1]	Mean thyroid gland iodine[2]	Mean serum protein-bound iodine, μg. %
			Original	Surviving				
1	21–26° C.	7		5	5.6	7.9 ± .64		2.8
				5	1.6	10.5 ± .99		2.9
				5	2.3	9.1 ± .38		3.5
	Mean		15	15	3.2	9.2 ± .47		3.1
	1– 7° C.	7		6	1.0	10.4 ± .98		2.7
				6	−3.5	12.7 ± .61		2.5
	Mean		15	12	−1.2	11.6 ± .65		2.6
3	26–31° C.	14	6	6	3.2	7.1 ± .99	18.5	2.9
	1– 7° C.	14	6	6	0.5	10.4 ± 1.10	19.5	2.4
2	21–26° C.	26		7	2.2	7.3 ± .57	35.0	3.1
		27		7	2.3	6.5 ± .45	47.0	3.3
	Mean		14	14	2.2	6.9 ± .36	41.0	3.2
	1– 7° C.	26		6	1.2	9.4 ± .35	44.0	2.6
		27		7	0.2	11.3 ± 1.36	45.0	3.0
	Mean		14	13	0.7	10.4 ± .59	44.5	2.8

[1] Mg. per 100 g. body weight ± standard error of the mean.
[2] Mg. iodine per 100 g. wet tissue.

RESULTS

1. *Effects of a cold environment on normal rats*

Table 1 records the data obtained in three experiments in which a total of 70 rats was used. Of these animals approximately half were kept in a warm (either 20.8 to 25.6° C. or 25.6 to 31.2° C.) and half in a cold (1.1 to 6.7° C.) environment. The duration of the study in experiment 1 was seven days, in experiment 3 fourteen days and in experiment 2 twenty-six or twenty-seven days.

The rats exposed to a cold environment had slightly, but consistently, lower concentrations of protein-bound iodine in the serum, and heavier thyroid glands than similar animals living in a warm room. However the concentrations of iodine in the thyroid were approximately equal. The "t" test for paired data was applied to the series of differences obtained by

subtracting the mean for the warm rats from the mean for the cold rats in each of the three experiments. For the serum protein-bound iodine the mean difference between the means was $-0.467\mu g.\ \%$ with a standard error of $\pm 0.033\ \mu g.\ \%$. This gave a "t" of 14.0, and for two degrees of freedom $P = <0.01$. The corrresponding mean difference for the thyroid weights was 3.066 mg. per 100 g. of body weight with a standard error of ± 0.338 mg. per 100 g. body of weight. This gave a "t" of 9.1, and for two degrees of freedom, $P = <0.05$ but >0.01. These differences are therefore probably significant. The differences noted were evident in all three experiments.

TABLE 2. EXPERIMENT 3. RATS FED PROPYLTHIOURACIL AND VARIOUS DOSES OF THYROID FOR 14 DAYS IN WARM AND COLD ENVIRONMENTS

Environmental temperature	Treatment		No. of animals		Mean daily weight gain, grams	Mean thyroid gland weight[3]	Mean thyroid gland iodine[4]	Mean acinar cell height[5]	Mean serum protein-bound iodine, $\mu g\ \%$
	Thyroid[1]	Propylthiouracil[2]	Original	Surviving					
26–31° C.	0	0	6	6	3.2	7.1± .99	18.5	7.6± .25	2.9
	1.7 mg.	+	7	7	2.6	18.6±2.42	1.1		1.1
	3.4 mg.	+	7	7	2.0	8.1±2.12	6.8	6.8± .18	1.6
	6.8 mg.	+	7	7	1.5	5.3± .42	18.5	4.4± .14	2.6
	13.6 mg.	+	7	7	1.3	5.4± .39	22.5	4.9± .16	2.5
1–7° C.	0	0	6	6	0.5	10.4±1.10	19.5	9.7± .24	2.4
	1.7 mg.[6]	+	7	1	0.6	23.8	5.6		1.4
	3.4 mg.	+	7	5	−0.3	23.2±2.07	1.3	10.6± .29	0.9
	6.8 mg.	+	7	5	−0.5	14.6±1.96	2.9	8.1± .28	1.5
	13.6 mg.	+	7	3	−0.9	7.5±1.27	10.5	5.6± .11	1.8

[1] Mg. per 100 g. body weight per day.
[2] 0.1% in food.
[3] Mg. per 100 g. body weight ± standard error of the mean.
[4] Mg. per 100 g. wet tissue.
[5] Microns ± standard error of the mean.
[6] These data omitted from Fig. 1. See comments in text.

2. *Effects of propylthiouracil and various doses of desiccated thyroid on rats exposed to warm and cold environments*

The results of these studies are given in Table 2 and Figure 1. Except for the single animal receiving the 1.7 mgm. dose of thyroid, all the groups of rats living at 1.1 to 6.7° C. had lower concentrations of protein-bound iodine in the serum, lower concentrations of total iodine in the thyroid gland, higher mean acinar cell heights and greater thyroid gland weights than similarly treated animals living at 25.6 to 31.2° C. The single animal given the 1.7 mgm. dose of thyroid and surviving exposure to cold had slightly higher values for serum protein-bound iodine and thyroid iodine than animals given the same dose in a warm environment. However, the fact that only one rat survived from an original group of seven suggests that in this animal the propylthiouracil block may not have been as complete as in the rats which succumbed to cold. The data obtained from this animal have therefore been omitted from Figure 1.

In Figure 1 the results of these experiments have been plotted as a series of dose response curves. The doses have been plotted on a logarithmic scale, and the mean responses for both warm and cold groups have been plotted as per cent of the mean values for the untreated controls living in a warm environment. Figure 1 reveals that approximately 6 mgm. of thyroid per 100 gm. of rat per day were needed to maintain normal values

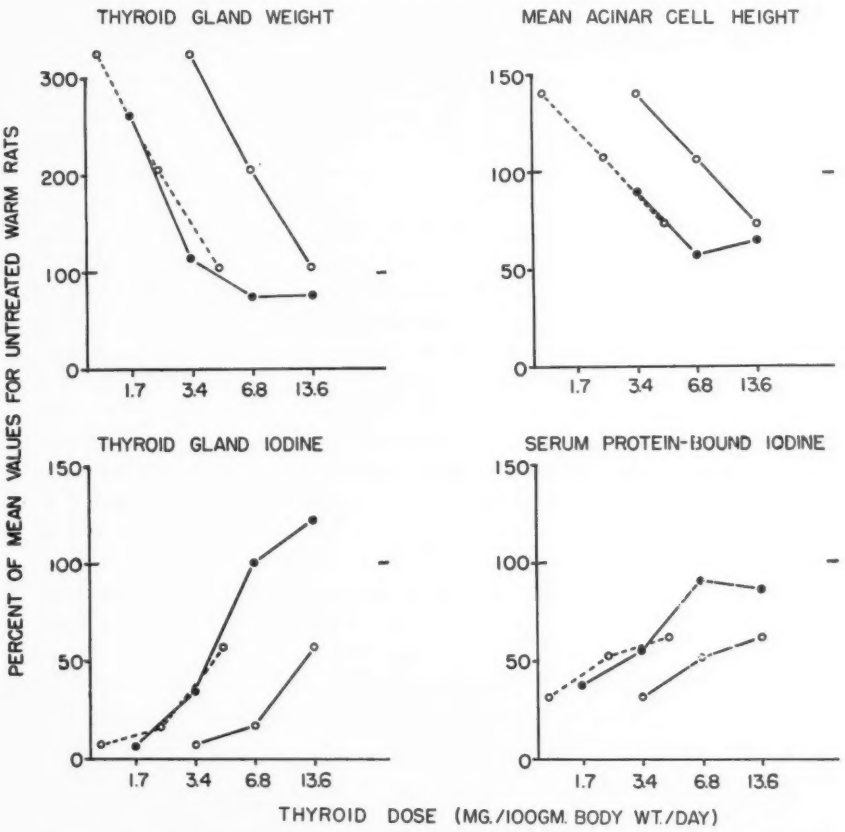

FIG. 1. The effects of various doses of desiccated thyroid on thyroid activity in rats treated with propylthiouracil and exposed to a warm environment (solid circles, solid lines) or to a cold environment (open circles, solid lines). The curves for rats exposed to a cold environment have also been shifted to the left by 0.48 log unit and replotted (open circles, broken lines) so as to demonstrate their similarity in shape and slope to the curves for rats in a warm environment. Abscissa: daily dose of U.S.P. Thyroid (milligrams per 100 grams of body weight) on a logarithmic scale. Ordinate: response expressed as per cent of the mean value for untreated control rats in a warm environment.

for serum protein-bound iodine, thyroid iodine, and thyroid weight of rats living at 25.6 to 31.2° C. In the rats exposed to cold, the values for serum protein-bound iodine and thyroid iodine were subnormal even when the dose of thyroid was 13.6 mgm. per 100 gm. of body weight per day. However this dose did maintain normal thyroid weight. In both warm and cold environments the mean acinar cell height was kept normal by comparatively small doses of thyroid and was actually diminished by the larger doses.

Although exposure to cold markedly decreased the responses to a given dose of thyroid, it did not significantly alter the shapes and slopes of the dose response curves so far as can be judged from the available data. Furthermore the lateral displacement of the curves for the rats exposed to cold was approximately 0.48 log unit for each of the four responses studied. In order to emphasize these facts, in Figure 1 each of the curves for the rats exposed to cold has been shifted to the left by 0.48 log unit and replotted as a broken line. In every instance the shifted lines practically coincide with, or constitute a reasonable extension of the dose response curves for the rats in a warm environment. Since 0.48 log unit corresponds to a factor of 3, it is evident that the requirement for thyroid hormone increased about threefold in the animals kept at a temperature of 1.1 to 6.7° C. Dempsey and Astwood (1943) using similar experimental conditions reported an increase of approximately the same magnitude in the daily dose of l-thyroxine given hypodermically which was required for the prevention of thyroid enlargement in rats treated with thiouracil.

COMMENTS

The literature contains extensive evidence suggesting that exposure of rats to cold leads to considerable increases in (a) production of thyroid stimulating hormone by the anterior pituitary, (b) thyroid gland activity, and (c) utilization of thyroid hormone by body tissues (Dempsey and Astwood, 1943). In addition there occurs some increase in the basal metabolic rate, an increase, however, which is due only in small part to augmented activity of the thyroid gland (Ring, 1936). While this information reveals the thyroid to be an organ which undergoes homeostatic fluctuations in secretory activity in response to a changing environmental circumstance, it fails to indicate how cold induces these changes.

Two possible mechanisms come to mind. In one it may be postulated that exposure to cold leads primarily to increased production of thyroid stimulating hormone. This in turn would be expected to augment the rate of production of hormone by the thyroid gland, a phenomenon which should be followed by an elevation in the concentration of thyroid hormone in the blood stream as measured by the protein-bound iodine. Finally, the

elevation in circulating thyroid hormone might be expected to result in increased rates of tissue energy metabolism (Dempsey and Astwood, 1943) Alternatively, it may be proposed that the primary effect of cold so far as the thyroid mechanism is concerned is to cause an increase in the rate of utilization of thyroid hormone by body tissues. This, by drawing hormone from the circulation, would tend to lower the concentration of thyroid hormone in the blood stream. The effect of this lowering would be to prompt a compensatory increase in the secretion of thyroid stimulating hormone and hence in the secretory activity of the thyroid gland.

The observations described here support the second of these two hypotheses. Our data indicate that exposure to cold definitely does not result in the increase in concentration of hormonal iodine in the serum necessary for fulfillment of the first hypothesis. On the contrary, cold leads to a slight but probably significant* decrease in serum protein-bound iodine. Furthermore as revealed by the studies of serum protein-bound iodine in rats treated with propylthiouracil and thyroid, an increase in thyroid hormone utilization upon exposure to cold occurs even when the concentration of thyroid hormone in the blood stream is subnormal. In other words, these observations strongly suggest that the primary effect of cold is to increase the peripheral utilization of thyroid hormone. In the normal animal the anterior pituitary compensates for this increased utilization by stimulating thyroid activity just enough to maintain a practically normal concentration of thyroid hormone in the circulation. When thyroid activity is blocked by propylthiouracil the increased quantity of exogenous hormone required in the cold environment for suppression of pituitary hyperactivity and for maintenance of a *normal* concentration of circulating thyroid hormone is a clear indication of the increased demands of the peripheral tissues.

In evident conflict with this hypothesis is Uotila's observation that section of the pituitary stalk in rats will prevent hypertrophy of the thyroid due to exposure to cold, but will *not* interfere with the compensatory hypertrophy of a thyroid remnant after subtotal thyroidectomy (Uotila, 1940). This observation suggests that the response of the pituitary and thyroid to cold is not a simple compensatory response but is dependent upon a primary stimulus reaching the hypophysis via the pituitary stalk. At present there seems no way in which our observations can be reconciled with those of Uotila. However Dempsey and Searles (1943), while confirming Uotila's results with cold exposure, have also demonstrated that stalk section in no way interferes with the thyroid atrophy which occurs

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* We are indebted to Dr. Jane Worcester of the Department of Biostatistics of the Harvard School of Public Health for a statistical appraisal of this point.

when rats are exposed to very warm temperatures. It is difficult to believe that the adaptive response of the thyroid gland to changes in environmental temperature depends upon two entirely different mechanisms, one for cold and one for heat.

Our studies have implications concerning the nature and purpose of the pituitary-thyroid system as a homeostatic mechanism. They suggest strongly that this system normally attempts to maintain the concentration of thyroid hormone in the circulation within physiologically optimal limits by adjusting the rate of hormone production to changing bodily needs. In healthy human subjects (except during pregnancy) these physiologic limits are indicated by the normal range for serum protein-bound iodine of about 4 to 8 microgm. per cent. Interestingly, deviations above or below this range almost always are accompanied by pathologic manifestations of hyper- or hypothyroidism. Studies of the growth and maturation of children fail to suggest that any normal growth or maturation process is ascribable to a change in the concentration of thyroid hormone to which the tissues are exposed such as occurs in the concentration of sex hormones during adolescence (Talbot *et al.*, 1952). It appears that body cells function best when bathed in blood containing thyroid hormone at concentrations which fall within certain limits just as they do when the pH and the sodium, potassium, calcium and phosphorus concentrations of the serum are within normal limits. This describes the thyroid hormone more as a substance essential for the *support* of normal cell activity than as a circulating messenger designed to *alter* the functional activity or development of other organs. While other endocrine glands vary their rate of secretion so as to regulate the activity of specific target organs or to maintain the constancy of certain non-endocrine components of the internal environment, the thyroid gland behaves as though its secretion were *itself* an essential part of the internal environment.

SUMMARY

The effects of exposure to cold upon the concentration of protein-bound iodine in the thyroid gland and serum and upon the weight and mean acinar cell height of the thyroid glands of normal rats and of rats given propylthiouracil and various doses of desiccated thyroid are described. The tendencies to thyroid enlargement and to increased need for thyroid hormone described by others as incident to cold exposure are confirmed. In addition it is shown that cold fails to lead to an elevation and probably occasions a slight decrease in the concentration of thyroid hormone in the circulation of normal rats as indicated by the serum protein-bound iodine. These findings are interpreted to mean that the primary effect of cold is to induce an increase in the utilization of thyroid hormone by the peripheral

tissues. This tends to decrease the concentration of thyroid hormone in the blood stream, a change which normally prompts a compensatory homeostatic increase in the production of thyroid stimulating hormone and hence in the secretory activity of the thyroid gland. Some of the implications of these observations with relation to the role of the thyroid in body economy are discussed briefly.

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NOTES AND COMMENTS

SYNERGISTIC AND ANTAGONISTIC EFFECTS OF PROGESTERONE IN COMBINATION WITH ESTROGENS ON OVIDUCT WEIGHT[1,2]

Synergistic effects of progesterone when injected with estrogens on the oviduct weight of pullets has been described by Mason (1952) but an antagonistic action of progesterone on oviduct growth when stilbestrol was administered has also been reported by Hertz, Larsen, and Tullner (1947). It was suggested by Mason (1952) that these conflicting data might be due to the variation in the dosage levels of the hormones. In view of this suggestion, it was felt that it would be desirable to carry out an experiment using a single group of chicks receiving both high and low levels of either estradiol or stilbestrol in combination with progesterone.

MATERIALS AND METHODS

Single comb White Leghorn pullets were injected daily with various dosages of hormones[3] from the 23rd to the 32nd day of age inclusive and were autopsied when they were 33 days old. All hormones were administered in separate subcutaneous injections of 0.05 cc. of sesame oil, hence animals which received two injections received 0.10 cc. of carrier. Control series were given 0.05 cc. of sesame oil. The oviducts were dissected free from the surrounding tissue at the time of autopsy, weighed, and fixed immediately in 10% formalin. All weights were obtained on either a Roller Smith torsion balance or a beam balance according to the range of weights for the oviducts of that particular series.

RESULTS AND DISCUSSION

The administration of both estradiol and stilbestrol caused a marked increase in oviduct weight and this increase was correlated with the dosage of the hormones. Stilbestrol, however, was not as effective as estradiol in producing weight increases. Administration of 200 μg. of stilbestrol and estradiol resulted in mean oviduct weights of 1587 mg. and 1985 mg. respectively. Progesterone alone had no effect on oviduct weight and this observation confirms the results described by Hertz, Larsen, and Tullner (1947) and Mason (1952). Five hundred μg. per day of progesterone, however, had a stimulating effect on oviduct weight in the presence of 20 μg./day of either estradiol or stilbestrol, increasing the oviduct weight from 129 mg. to 229 mg. and 166 mg. to 597 mg. respectively. The augmenting effect of progesterone, on the other hand, was reversed when the dosage of either estradiol or stilbestrol was increased to 200 μg./day. This action of progesterone resulted in a decrease in oviduct weight from 1587 mg. for stilbestrol alone to 974 mg. for for the combination of stilbestrol and progesterone. A decrease was also observed in the

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[2] In partial fulfillment of the requirements for the degree, Doctor of Philosophy, Graduate School, Indiana University.

[3] The estradiol dipropionate used in this experiment was provided through the courtesy of Dr. Robert Gaunt of Ciba Pharmaceutical Products, Summit, New Jersey; the progesterone and diethylstilbestrol were provided through the courtesy of Dr. K. K. Chen of the Eli Lilly Company, Indianapolis, Indiana.

combination of 500 μg./day of progesterone and 200 μg./day of estradiol, but in this instance the decrease was not statistically significant.

These results confirm the previously published reports that progesterone both augments and inhibits the action of estrogens on the oviduct. Adequate dosages of progesterone in combination with low doses of estrogens caused a hypertrophy of the oviduct as reported by Mason (1952) but progesterone in combination with large doses of estrogens caused a decrease in oviduct weight (Hertz, Larsen and Tullner, 1947). The results for the 50 μg./day dosage of progesterone show that the response to progesterone in combination with the high dosage of estrogens was not a simple hormone antagonism. The 50μg./day dosage of progesterone in combination with 200 μg./day of stilbestrol

TABLE 1

Treatment in μg/day		Mean oviduct weight in mg.	Number
Estrogen	Progesterone		
0	0	45 ± 3*	10
0	500	48 ± 1	15
Stilbesterol			
20	0	130 ± 11	14
20	50	141 ± 8	13
20	500	229 ± 15	15
200	0	1587 ± 75	15
200	50	2273 ± 91	15
200	500	974 ± 65	15
Estradiol†			
20	0	166 ± 15	15
20	500	598 ± 52	14
200	0	1985 ± 170	13
200	500	1629 ± 96	14

* Standard Error.
† Estradiol dipropionate.

caused a marked increase in oviduct weight but the same amount of progesterone in combination with only 20 μg./day of stilbestrol had little effect. These data demonstrate in a striking manner the problem of hormone interactions and emphasize the complexity of this problem.

The data reported here furnish information on whether progesterone operates to augment or depress the growth promoting effects of estrogen on the oviducts of untreated birds. It has been shown, for example, by Breneman (1942) and Breneman and Mason (1952) that estrogens at a level corresponding to the high dosage level of estrogens used in this experiment will completely inhibit pituitary activity. It would appear that endogenous estrogens are not present normally at a level equivalent to the 200 μg./day dosage as used in this experiment. The endogenous progesterone in untreated birds must be operating in conjunction with much lower levels of estrogens and must, therefore, augment the effects of endogenous estrogens on the oviduct.

SUMMARY AND CONCLUSIONS

Data are presented that show that 500 μg./day of progesterone in combination with 20 μg./day of either stilbestrol or estradiol will cause a hypertrophy of the oviducts of

White Leghorn pullets over that produced by estrogens alone. This effect of progesterone, on the other hand, is reversed when administered in combination with 200 μg./day of either stilbestrol or estradiol. When, however, 50 μg./day of progesterone was given in combination with the high dose of estrogens, there was a significant increase in oviduct weight. These data emphasize the importance of the relative dosages of estrogens and progesterone in determining the physiological response to these hormones.

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ADRENAL CORTICAL ACTIVITY IN SCURVY

It has been shown earlier that both cholesterol and ascorbic acid concentrations of adrenals are diminished in scorbutic guinea pigs (Banerjee and Deb, 1951). It has also been observed that a scorbutic female guinea pig excretes less 17-ketosteroids in urine than its paired-fed control (Banerjee and Deb, 1952). Based on the above observations it has been suggested that adrenal cortical activity is diminished in scurvy. The adrenal cortex is also related to the metabolism of sodium and potassium in the body. After removal of the adrenals there is a decreased sodium content and an increased potassium content of blood and a diminished urinary excretion of potassium (Baumann and Kurland, 1927). Sodium chloride has a beneficial effect in adrenalectomized animals (Hartman, *et al.* 1927). In order to present further evidences regarding the activity of the adrenal cortex in scurvy sodium and potassium content of serum and urinary excretion of potassium have been estimated in both normal and scorbutic guinea pigs. As increased concentration of potassium in blood depresses the cardiac and plain muscles of the body, the effect of addition of serum obtained from normal and scorbutic guinea pigs on the contraction of toad's heart has been recorded. The rhythmic contractions of the uterus from both normal and scorbutic guinea pigs have also been recorded.

MATERIAL AND METHODS

Female guinea pigs were selected and paired as described previously (Banerjee and Deb, 1951). Animals were placed in separate metabolism cages and urines were collected under toluene. Potassium in the 24 hours' output of urine, collected on the 21st day of the experiment, was estimated by the method of Abul-Fadl (1949) described for the estimation of potassium in blood. By recovery experiments it was observed that the method was suitable for the estimation of potassium in urine. On the 21st day of the experiment blood was collected by cardiac puncture and in the serum potassium was estimated by the method of Abul-Fadl (*loc. cit.*) and sodium by the method of Weinbach (1935). The results are shown in Table 1.

Toads with an average weight of 100 gm. were perfused (Bain, 1938) with Ringer's fluid and the contractions of the heart were recorded on a smoked drum. 0.2 cc. of serum of the

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TABLE 1. DETERMINATION OF SODIUM AND POTASSIUM IN BLOOD AND POTASSIUM IN URINE OF NORMAL AND SCORBUTIC GUINEA PIGS

Pair No.	Weight at death	Potassium in 24 hour urine	Potassium in 100 cc. serum	Sodium in 100 cc. serum
	gm.	mg.	mg.	mg.
1. Normal	250	95.7	18.0	290.2
Scorbutic	220	50.0	35.3	169.8
2. Normal	272	125.1	21.6	266.8
Scorbutic	250	73.2	41.9	200.1
3. Normal	266	103.2	20.9	232.5
Scorbutic	215	68.6	46.2	187.6
4. Normal	235	98.7	26.2	240.0
Scorbutic	211	58.9	38.2	193.3
5. Normal	261	112.0	15.0	273.7
Scorbutic	226	64.0	42.1	202.4
6. Normal	227	109.8	16.3	260.9
Scorbutic	189	71.5	45.7	170.3
7. Normal	230	111.6	23.7	251.6
Scorbutic	190	73.7	36.5	185.4
8. Normal	238	108.1	20.8	285.6
Scorbutic	215	94.2	38.8	210.0
Average				
Normal		108.0 ±3.3	20.3 ±1.3	262.6 ±7.4
Scorbutic		69.2 ±4.6	40.5 ±1.7	189.8 ±5.4
Difference of means		38.8	20.2	72.8
Standard error of difference		5.68	2.16	9.19
t		6.8	9.3	7.9

All values of *t* are high significant.

guinea pigs, either normal or scorbutic, was added into the perfusing fluid and the contractions of the heart were recorded. The results are shown in Figures 1 and 2.

On the 21st day of the experiment the scorbutic guinea pigs with their normal controls were killed by a blow on the head followed by decapitation. The uterus was dissected out and one horn of the uterus from each of the animals was suspended in separate organ bath from which simultaneous contractions from two uteri, one scorbutic and the other normal, could be recorded was set up in the usual way and the contractions were recorded. The results are shown in Figure 3.

RESULTS

In scorbutic guinea pigs there was a significant decrease in the sodium content and an increase in the potassium content of the blood. Scorbutic guinea pigs excreted less potassium in urine. The amplitude of contractions of a toad's heart was depressed when the heart was perfused with the serum from a scorbutic animal. The normal contractility of the uterus of a scorbutic guinea pig kept in an isolated organ bath was found depressed.

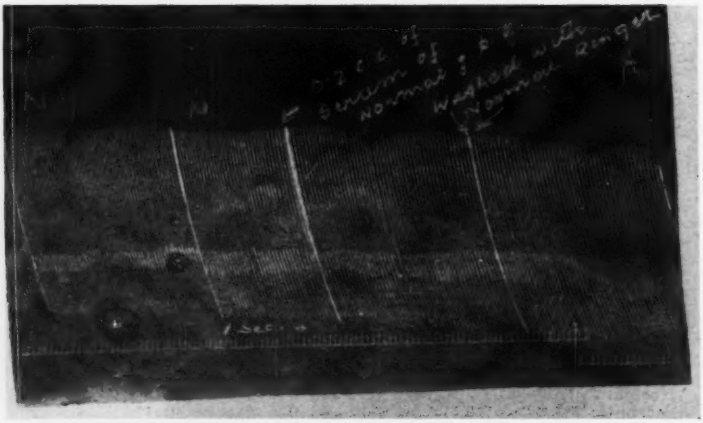

FIG. 1. Showing effect of normal serum on the contraction of a toad's heart perfused with Ringer's solution containing 0.2 cc. of serum obtained from the blood of a normally fed guinea pig.

DISCUSSION

Giroud *et al.* (Giroud, Santa and Martinet, 1940; Giroud, Santa, Martinet and Bellon, 1941) suggested a decrease in the adrenal cortical hormone in scorbutic guinea pigs without, however, furnishing sufficient biochemical data. A decrease in the adrenal cholesterol and adrenal ascorbic acid has also been suggested to be due to a hypersecretion of the adrenal cortical hormone as a result of the pituitary adrenocorticotrophic hormone (Sayers, 1950). The decreased liver glycogen values of scorbutic guinea pigs

FIG. 2. Showing the effect of perfusion of a toad's heart with Ringers' solution containing 0.2 cc. of serum obtained from the blood of a scorbutic guinea pig.

and a diminution in the urinary excretion of 17-ketosteroids by scorbutic female guinea pigs, however, not only do not give support to the hypersecretion of the adrenal cortex but indicate its hypoactivity in scurvy. The diminished sodium content of serum of scorbutic guinea pigs as observed by us is, however, contradictory to the observations of Stepto *et al.* (Stepto, Pirani, Consolazio and Bell, 1951) who found normal blood sodium values in scorbutic guinea pigs. Evidences brought forward by us indicate that both

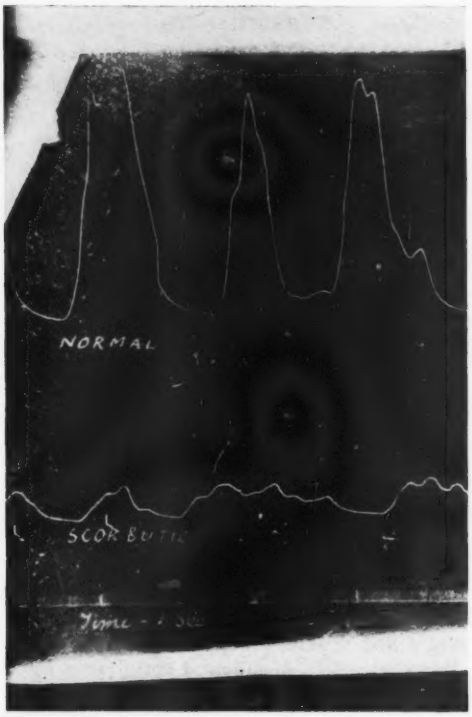

FIG. 3. Showing the rhythmic contractility of uterus from a normal and a scorbutic guinea pig.

the salt regulating and the carbohydrate metabolism regulating hormones of the adrenal cortex are diminished in scorbutic guinea pigs. This diminished formation of adrenal cortical hormone in scurvy might be due to the decreased ascorbic acid and cholesterol contents of the adrenal glands of a scorbutic animal which have been suggested to be the raw materials for the synthesis of the adrenal cortical hormone.

SUMMARY

Sodium and potassium contents of blood and the urinary excretion of potassium were determined in scorbutic and paired fed normal guinea pigs. Scorbutic animals had increased potassium and decreased sodium content of the blood. Scorbutic animals excreted less potassium in urine. Serum from scorbutic animals diminished the amplitude

of contractions of a toad's heart and the rhythmic contractility of an isolated uterus from a scorbutic guinea pig in an organ bath was depressed. Scorbutic guinea pigs seem to suffer from hyposecretion of the adrenal cortex.

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ASCORBIC ACID AND FORMALDEHYDE-IRRITATION ARTHRITIS IN THE ADRENALECTOMIZED RAT

Previous work showed that ascorbic acid enhances the recovery of intact (i.e. non-adrenalectomized) rats with formaldehyde-irritation arthritis (Bacchus, 1951). Since it had been observed that the protective action of the vitamin against stressors may be dependent on the presence of intact adrenals or of its secretions, it seemed desirable to examine the action of ascorbic acid in the adrenalectomized rat. This report presents data indicating that ascorbic acid fails to enhance the recovery of adrenalectomized rats with formaldehyde-irritation arthritis.

MATERIALS AND METHODS

Female albino rats weighing about 220 grams were used in this study. The animals were bilaterally adrenalectomized under ether anesthesia at least five weeks prior to experimentation, and maintained on an ad libitum fare of Purine Laboratory Chow and 0.9% NaCl. At the beginning of the experiment all animals were injected with 0.1 ml. of 2.5% formaldehyde into the palmar aponeurosis of one hind leg, the other hind leg serving as the untreated control. Measurements of the volume of each leg were made just prior to the injection, and once each day subsequent to the injection. The volume of the leg was measured according to the method used previously; the method involved the displacement of mercury from an overflow cup (Bacchus, 1951). The amount of swelling was calculated from the volume measurements as the difference between the *change* of size of each leg when compared with its size on the first day (Bacchus, 1951).

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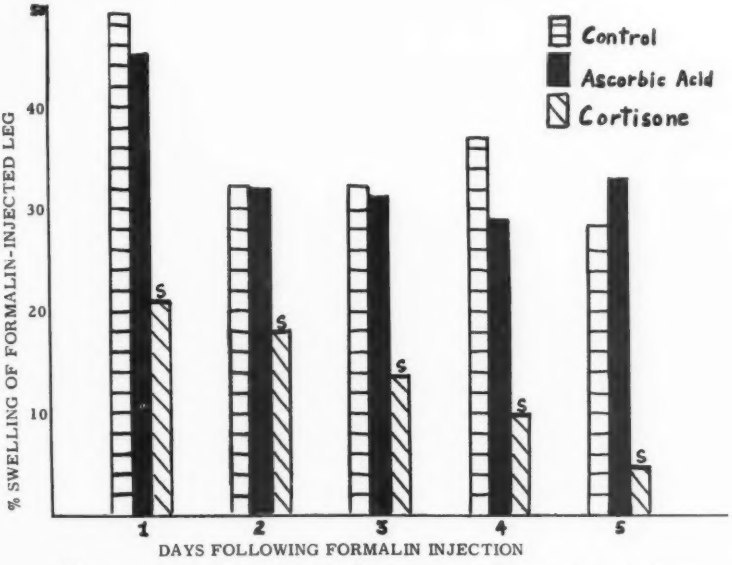
Group I consisted of six adrenalectomized rats which received, from the beginning of the experiment, 75 mgm. of ascorbic acid (Na ascorbate, Roche) per 100 gm. body weight, in single intraperitoneal injections. Group II consisted of four adrenalectomized rats which received daily subcutaneous injections of cortisone acetate (Merck) at the rate of 3 mg./100 gm. Group III consisted of six adrenalectomized rats which received no injectional therapy; these served as controls for the above groups.

RESULTS

In Figure 1 the per cent increase in the size of the legs are presented, the statistically significant differences being indicated by "S." It is observed that the swelling is very marked in the untreated animals throughout the period of study. The swelling in the



Mean percentages are presented ; S = difference from controls is significant.

FIG. 1

cortisone-treated rats is significantly less than that in the control animals; this difference was statistically significant ($P = < 0.01$) throughout the experiment. Ascorbic acid, on the other hand, did not diminish the swelling due to formaldehyde-irritation. Gross examination of the legs indicated that the swelling and edematous appearance of the formaldehyde-injected legs of Groups I and III rats were present even on the fifth day of observations. These gross changes disappeared by the second day in the cortisone-treated rats.

DISCUSSION

The data indicate that ascorbic acid treatment to adrenalectomized rats with formaldehyde-arthritis fails to reverse the swelling and edematous changes. This is in marked contrast to the observation that the vitamin, in similar doses as these, enhances the recovery in intact (i.e. non-adrenalectomized) rats (Bacchus, 1951). The data suggest

that the anti-arthritis action of ascorbic acid is not direct and may involve the participation of cortical hormones. While the exact mechanism of this participation is not known, the following facts may have a bearing on it. It is highly unlikely that the action is due to a stimulation of the pituitary-adrenal axis by the vitamin (see also Bacchus, 1951). Similarly it is unlikely that the vitamin increases the production of adrenal cortical hormones (Bacchus, Heiffer and Altszuler, 1952; Stepto *et al.*, 1951). Data have been obtained in this laboratory indicating that the excretion of the 17-ketosteroid metabolites of cortisone is decreased by simultaneous ascorbic acid treatment. Associated with this decreased excretion was a prolongation, for a corresponding period, of the hematologic actions of cortisone by ascorbic acid (Bacchus, Altszuler and Heiffer, 1952). Certain preliminary data suggest that this action of ascorbic acid is not due to a reconversion of 17-ketosteroid metabolites to active cortical steroids, but rather due to a diminished breakdown of cortisone.

The data of the present study are in consonance with the suggestion that ascorbic acid depresses the inactivation of glucocorticoid hormones. The "maintained" levels of these hormones, therefore, exert their well known anti-arthritic actions.

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This work was supported by a grant from the National Institutes of Health. The ascorbic acid (Na ascorbate, Roche) was obtained through the generosity of Dr. R. A. Lewis, Hoffman-La Roche, Nutley, New Jersey. Dr. E. Alpert, Merck and Co., Rahway, New Jersey, kindly supplied the Cortone used in this study.

THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be limited to ten minutes, should send FOUR COPIES of the title and abstract to the Vice-President, Dr. Lawson Wilkins, Johns Hopkins Hospital, Baltimore 5, Maryland, not later than FEBRUARY 1, 1953. IT IS IMPERATIVE THAT THE ABSTRACTS BE INFORMATIVE AND COMPLETE WITH RESULTS AND CONCLUSIONS IN ORDER THAT THEY MAY BE OF REFERENCE VALUE AND SUITABLE FOR PRINTING IN THE PROGRAM AND JOURNALS OF THE SOCIETY. THE FOLLOWING REGULATIONS FOR THE PREPARATION OF ABSTRACTS AND TITLES MUST BE CAREFULLY FOLLOWED TO INSURE CONSIDERATION OF THE PAPER FOR THE PROGRAM:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes nor acknowledgements to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-

authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.

Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter-perfect, since there will be no opportunity for proof reading by authors.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was

unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1953.

ANNOUNCEMENT

The University of Minnesota will present a continuation course in "Recent Advances in Diagnosis for Internists" at the Center for Continuation Study on February 16 to 18, 1953. Although intended primarily for specialists in internal medicine, the program will be of interest to many pediatricians and general physicians. This course will provide information concerning techniques which have been introduced or developed within the past five to ten years which have increased our knowledge of basic physiology and which are becoming or give promise of becoming standard diagnostic procedures. Recent developments in the fields of cardiology, respiratory disease, renal disease, hematology, gastro-enterology, and endocrinology will be emphasized.

Under the direction of Dr. C. J. Watson, Professor and Director of the Department of Medicine, an outstanding guest faculty has been assembled: Dr. Carl V. Moore, Professor, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri; Dr. Thomas E. Machella, Assistant Professor, Department of Medicine, and Associate in Physiology, University of Pennsylvania School of Medicine, and Chief of Gastro-Intestinal Clinic, Hospital of the University of Pennsylvania, Philadelphia; Dr. Robert P. Grant, U. S. Public Health Service Hospital, Baltimore; and Dr. William W. Engstrom, Associate Professor, Department of Medicine, Marquette University School of Medicine, and Director, Metabolism Section, Milwaukee County Hospital. The remainder of the faculty will include clinical and full-time members of the staff of the University of Minnesota Medical School and the Mayo Foundation.

Lodging and meal accommodations are available at the Center for Continuation Study.

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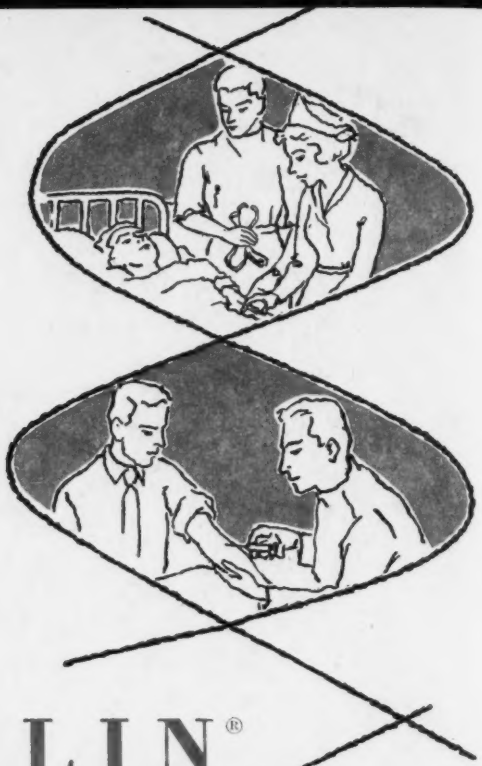

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